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Exploring the effect of NLRP3 Inflammasome inhibition in the *ex vivo* model of epileptogenesis

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2 RESUMO

De acordo com a *World Health Organisation*, a epilepsia é uma causa crescente de morbidade e mortalidade. Atualmente, a epilepsia é a terceira condição neurológica crónica mais comum em todo o mundo, com 50 milhões de indivíduos afetados e 2,4 milhões de novos casos diagnosticados em cada ano. A epilepsia é considerada um conjunto de distúrbios cerebrais que consiste, principalmente, em convulsões recorrentes, espontâneas e sintomáticas sem identificação imediata de causa. Durante os eventos epiléticos, o indivíduo torna-se incapaz de controlar conscientemente o seu próprio corpo. Por isso, a epilepsia é uma doença com um elevado fardo emocional tanto para os pacientes como para as suas famílias.

Epileptogénese é o processo pelo qual um cérebro saudável se altera e desenvolve epilepsia. Nos seres humanos, este processo é iniciado por fatores geneticamente herdados, lesões do sistema nervoso central e/ou infecções microbianas. Para induzir este processo em animais, os cientistas utilizam fatores que iniciam o *status epilepticus* (SE), definido como um período de convulsões contínuas com duração superior a 5 minutos. São vários os fatores utilizados nos modelos animais de epilepsia, mas os mais populares são as abordagens farmacológicas, com kainato ou pilocarpina, e protocolos específicos tais como o *kindling* ou a lesão cerebral. Nestes modelos ocorre um aumento da excitabilidade do sistema que desencadeia o desenvolvimento de convulsões. A morte neuronal, a ocorrência de neuroinflamação e a produção de citocinas pró-inflamatórias são consideradas marcadores moleculares da epilepsia. No entanto, os mecanismos exatos envolvidos no processo da epileptogénese não são ainda totalmente compreendidos.

A ativação das células da glia, nomeadamente dos astrócitos e da microglia, denominada de gliose, ocorre na maioria das desordens neurológicas, pelo que estas células têm sido alvo de vários estudos a fim de determinar o seu papel na patofisiologia de cada doença.

De facto, vários estudos têm vindo a demonstrar que tanto a microglia como os astrócitos, juntamente com os neurónios, apresentam um papel crucial na regulação da atividade epiléptica. Os astrócitos circundam os neurónios, tanto em condições fisiológicas como patológicas, e são considerados moduladores da atividade sináptica através da libertação de gliotransmissores. Em termos de morfologia celular, em locais de lesão ou infeção, estas células tornam-se activadas, o que corresponde a um aumento da proliferação celular e dos processos que emergem do corpo celular. Estas características são, frequentemente, designadas como uma cicatriz astroglial.

A microglia, consideradas as células imunes por excelência do sistema nervoso central, detetam e respondem a lesões ou infeções. Normalmente, na ausência de sinais patogénicos ou de perigo, a microglia percorre o cérebro em estado de repouso, monitorizando o ambiente e supervisionando a atividade neuronal e astrocítica. Neste estado, a microglia tem uma morfologia ramificada semelhante às células dendríticas do sistema imune inato. Após ativação destas células em resposta a lesões ou estímulos pró-inflamatórios, a sua morfologia celular muda rapidamente para um estado *primed*, caracterizado por um maior corpo celular e por processos curtos. Finalmente, quando estas células ficam ativas, adotam um corpo celular redondo e sem processos, referido como o estado amoebóide. Neste estado e em locais de lesão, as células da microglia apresentam a capacidade (tal como as células dendríticas) de libertar vários mediadores inflamatórios, principalmente citocinas.

Atualmente, várias publicações consideram a existência de uma interação entre neuroinflamação e epileptogénese, demonstrando um aumento de citocinas pró-inflamatórias, tais como a interleucina-1β (IL-1β), o factor de necrose tumoral alfa (TNF-α) e a interleucina-6 (IL-6). A IL-1β é processada pela enzima Caspase-1, que por sua vez é ativada pela formação de um complexo multiproteíco

denominado inflammassoma. Vários inflamassomas já foram descritos, sendo o inflamassoma NACHT, LRR, Pirina domínios-contendo proteína 3 (NLRP3), o mais conhecido. A ativação deste inflamassoma processa-se pela deteção de patogénios e/ou sinais endógenos de perigo. Depois da ativação, inicia-se o processo de formação do inflamassoma NLRP3 através da dimerização do NLRP3 ativo e posterior ligação com a proteína adaptora de domínio de recrutamento de caspase (ASC), que por sua vez interactua com a Caspase-1.

O objectivo deste estudo foi determinar se, num modelo *ex vivo* de epileptogénese, a inibição do inflamassoma NLRP3, utilizando um composto contendo diarilsulfonilureia, denominado MCC950, tinha efeito na neuroinflamação e na atividade epileptiforme. Este estudo utilizou um modelo de epileptogénese em fatias organotípicas de hipocampo/córtex entorrinal. Neste modelo, as fatias adquirem atividade epilética espontânea após 14 dias em cultura. Este tipo cultura apresenta várias vantagens em relação às culturas primárias, tais como o facto das células se desenvolverem de forma semelhante às células in vivo, de possuírem todos os tipos celulares (neurónios, astrócitos e microglia) e de poderem ser mantidas por longos períodos de tempo, permitindo, assim manipulação e avaliação a longo prazo. Deste modo, este modelo de epileptogénese em fatias organotípicas é um modelo simples que pode ser usado em ensaios de *screening* de potenciais compostos antiepiléticos.

As culturas foram preparadas a partir de ratos Sprague-Dawley com 6-7 dias de vida (P6-7). De acordo com o modo de manutenção das fatias, poder-se-á obter fatias com atividade epileptiforme (EL), se mantidas em meio de cultura sem soro, ou fatias que não apresentam atividade, se na presença de meio com soro, designadas como fatias controlo (CTR). Foi então estudada a influência do composto MCC950 (10μM), neste modelo de epileptogénese, a nível de morte celular, neuroinflamação e atividade epileptiforme. Os ensaios foram realizados em amostras obtidas a 7 e/ou 14 dias in vitro (DIV). O efeito do veiculo (0.1% DMSO) foi avaliado em todos os ensaios.

As diferenças na expressão de NLRP3 foram avaliadas em fatias EL e CTR a 14 DIV, visto ser neste dia que as fatias EL manifestam eventos interictais, não observados em fatias CTR. A morte celular, considerada um marcador molecular da epileptogénese, foi avaliada pela ocorrência da clivagem da αII-spectrina. Em caso de necrose, esta proteína (250 kDa) é clivada pela calpaína produzindo dois fragmentos estáveis de 150kDa (SBDP150) e de 145 kDa (SBDP145). Em caso de apoptose, a clivagem pela caspase-3 origina um produto de 120 kDa (SBDP120). A expressão da αII-spectrina e dos seus produtos de clivagem foi avaliada após incubação com o MCC950 (10μM). A neuroinflamação, outro marco importante da epilepsia, causa um aumento na expressão das proteínas "glial fibrillary acidic protein" (GFAP) e de "ionized calcium-binding molecule 1 (Iba1), marcadores de astrócitos e de microglia, respetivamente. Para além de um aumento de expressão, também ocorrem alterações na morfologia destas células, avaliadas por ensaios de imunohistoquímica, acoplados a fluorescência. As imagens foram obtidas no microscópio confocal em objetivas de 20x e 40x. O efeito do MCC950 na atividade epileptiforme foi avaliada por registos electrofisiológicos, na região CA3 do hipocampo, em fatias com 14 DIV.

Os resultados demonstraram que a expressão de NLRP3 foi mais elevada em fatias EL em comparação com fatias CTR que não apresentavam atividade. A expressão da αII-spectrina e dos seus produtos de clivagem foi avaliada após incubação com o MCC950 (10μM), durante um período de 6 horas, não se tendo observado qualquer efeito. Por outro lado, a exposição ao MCC950 (10 μM) causou diminuição na expressão de Iba1 e GFAP, e nas alterações de morfologia dos astrócitos e da microglia avaliadas através de microscopia confocal. Além disso, as fatias expostas a MCC950 não demonstraram atividade epileptiforme obtida por registos de potencial de campo espontâneos a partir da região CA3 do hipocampo. Assim sendo, o MCC950 parece silenciar as descargas epiléticas recorrentes presentes nas fatias EL.

Em resumo, este estudo demonstra que as fatias organotípicas com atividade epileptiforme sujeitas ao MCC950 (10 μM) exibem diminuições na astrogliose e microgliose, assim como uma atividade epileptiforme reduzida. No entanto, são necessários mais estudos para compreender melhor os mecanismos de ação do MCC950 e provar plenamente a sua eficácia como um fármaco antiepilético.

Palavras-chave:

Epilepsia, Epileptogénese, Neuroinflamação, Inflamassoma NLRP3, MCC950

3 ABSTRACT

Epilepsy is the third most common chronic neurological condition worldwide and is currently considered a growing cause of morbidity and mortality. It mainly consists of spontaneous and symptomatic recurring seizures with no immediate cause being identified. An association between neuroinflammation and epileptogenesis is well documented with studies now focusing on halting this disorder via potentially targeting the intracellular multi protein complex termed the inflammasome, composed by a pattern recognition receptor protein, an ASC domain and caspase-1. Various types of inflammasome have been discovered with the most well studied being the NACHT, LRR, Pyrin domains–containing protein 3 (NLRP3) inflammasome that is activated by the presence of pathogens and/or endogenous danger signals. Evidence suggests that inflammasome assembly occurs following this activation allowing for post translational processing of caspase-1, resulting in the processing of pro-IL-1β and subsequent production of IL-1β. This cytokine is released during seizures and is considered a pro-epileptogenic factor.

The aim of this study was to determine if inhibiting the NLRP3 inflammasome using a diarylsulfonylurea-containing compound, named MCC950, in an *ex vivo* model of epileptogenesis had any effect on neuroinflammation and epileptiform activity.

Results demonstrated that NLRP3 expression was higher in epileptic-like (EL) slices, compared to slices which did not depict epileptiform activity. Exposure to MCC950 (10μM) for a period of 6 hours caused lower Iba1 and GFAP expression, along with decreased alterations in astrocyte and microglia's morphology evaluated through confocal microscopy. Furthermore, slices exposed to MCC950 did not depict epileptiform activity, assessed by spontaneous field potential recordings from the CA3 region of the hippocampus. Thus, NLRP3 targeted inhibition appears to halt epileptic recurring discharges.

In summary, this study demonstrates that epileptic-like organotypic slices exposed to MCC950 (10μM) decreased astrogliosis and microgliosis, as well as a reduced epileptiform activity. However, further studies are needed to better understand its mechanisms of action and fully prove MCC950 efficacy as an anti-epileptic drug.

Key Words:

Epilepsy, Epileptogenesis, Neuroinflammation, NLRP3 Inflammasome, MCC950

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5 ABBREVIATIONS

Abbreviation	Term
Ab	Antibody
AC	Anti-convulsants
AEDs	Anti-epileptic drugs
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ASC	Apoptosis-related speck-like protein containing a caspase recruiting domain
BBB	Blood-brain-barrier
BSA	Bovine serum albumin
BSCB	Blood-spinal cord barrier
CA1	Cornus ammonis 1
CARD	Caspase recruiting domain
CNS	Central nervous system
CRID	Cytokine release inhibitory drug
CTR	Physiological normal like control model
CVA	Cerebrovascular accident
DAMP	Danger associated molecular pattern
DG	Dentate Gyrus
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
dsDNA	Double stranded Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EEG	Electroencephalogram
EL	Epileptic like model
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBSS	Gey's Balanced Salt Solution
GFAP	Glial fibrillary acidic protein
HMGB-1	High mobility group box-1
h	hours
HRP	Horseradish peroxidase

Iba1	Ionized calcium-binding molecule 1
IF	Immunofluorescence
IL-1β	Interleukin-1β
IL-1RAcP	Interleukin receptor protein-1 accessory protein complex
IL-1RI	Interleukin-1 receptor type 1
IL-1RII	Interleukin-1 receptor type 2
IL-18	Interleukin-18
K^+	Potassium
LPS	Lipopolysaccharides
LRR	Leucine rich repeat
MAPK	Mitogen-activated protein kinases
miRNA	Micro ribonucleic acids
MPC	Multi-protein-complex
MRI	Magnetic resonance imaging
MyD88	Myeloid differentiation factor 88
NACHT	Nucleotide-binding and oligomerization
NaCl	Sodium Chloride
NAD	NACHT associated domain
NALP3	NACHT, LRR and PYD domains-containing protein 3
NBA	Neurobasal A
NF-κB	Nuclear factor- κB
NLR	Nucleotide-binding oligomerization domain-like receptor
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NP40	Nonyl phenoxlpoylethanol
OHSC	Organotypic hippocampal slice culture
P	Probability
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer solution
PBST	Phosphate buffer solution with 0.1% Tween
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PN	Post-natal
Pro-IL-1β	Pro-interleukin-1β

PRR	Pattern recognition receptor
PNS	Peripheral nervous system
PYD	Pyrin domain
RIPA	Ristocetin Induced Platelet Agglutination
RT	Room Temperature
SD	Sprague Dawley (rats)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Status Epilepticus
SEM	Standard error of the mean
SBDP	Spectrin breakdown products
SiRNA	Short interfering ribonucleic acids
TBI	Traumatic brain injury
TLR	Toll-like receptor
TUNEL	Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling
WB	Western blot

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8 INTRODUCTION

8.1 NERVOUS SYSTEM

The nervous system has three main functions: sensory input, data integration and motor output. The system comprises mainly of structural cellular units called neurons. These operate via generating and propagating electrical signals via action potentials. Neurons facilitate communication between different regions of the nervous system since they provide the ability to amplify or modulate signals between themselves (inter-neurons) or with a non-neuronal cell, such as a skeletal muscle cell. This communication relies on electrical impulses or chemical signals, the latter being the role of signalling molecules referred to as neurotransmitters. Neural tissues also contain a type of non-neuronal cell, called glia. These function to ensure signal transmission efficiency, neuroprotection (to prevent damage caused by pathogens and endogenous danger signals) along with aiding in neuronal development and maintenance (Awasaki and Ito, 2004).

The nervous system is generally described as incorporating two major parts being the central nervous system (CNS) and peripheral nervous system (PNS). The CNS consists of the brain and spinal cord (Misra et al., 2003), while the PNS includes the somatic and autonomic systems (Navarro et al., 2005). Somatic processes are processes involving involve conscious voluntary control (Tsakiris and Haggard, 2003), whereas autonomic processes are not consciously controlled, such as breathing and heart rate (Pumprla et al., 2002).

Disorders of the brain and nervous system are common worldwide due to increasing life expectancy derived from life quality improvements. Many of these disorders have the ability to affect memory along with prohibiting an individual´s ability to go about his or her daily routines (Czira et al., 2014).

8.2 EPILEPSY

According to the World Health Organisation, epilepsy is a growing cause of morbidity and mortality and is currently the third most common chronic neurological condition worldwide (50 million affected individuals) with an estimated 2.4 million new cases being diagnosed each year (Prilipko et al., 2005). It is an associated group of brain disorders that mainly consist of spontaneous and symptomatic recurring seizures with no immediate cause being identified (Banerjee et al., 2009; Prilipko et al., 2005). These episodes can vary from being brief and nearly undetectable to demonstrating vigorous shaking during long periods of time (Edye et al., 2014). During the duration of these events the individual is rendered the inability to consciously control his or her own body. This disorder is also characterized by the onset of cognitive impairments and behavioural problems, such as isolation and overprotection (Masao and Kanemura, 2013). Thus, affected individuals tend to have an immense emotional burden on their families.

In several neurodegenerative diseases, such as Alzheimer's and Parkinson disease, the onset is mostly in middle aged or elderly individuals. However, people of all ages, sex and ethnicity are susceptible to epilepsy (Cloyd et al., 2006; Diederich et al., 2003; Vendrame et al., 2009). Even though there are no restrictions, ageing is considered to be a great risk factor for various diseases including those located in the CNS (Niccoli and Partridge, 2012). Currently the recorded incidence of this disorder is increasing and this is believed to be due to several factors. These include better detection techniques, inadequate primary health care, better life style quality, environmental exposures and ageing (Banerjee et al., 2009; Carlson et al., 2014; No et al., 2011; Theodore et al., 2006).

Generally, most cases of epilepsy are idiopathic with the rest of these being acquired as a result of a type of CNS injury (Prilipko et al., 2005). The first occurrence may involve genetically inherited factors (Frankel, 2009) while the latter may involve stroke, brain tumours, microbial CNS infections and traumatic brain injury (TBI) as a result of car collisions, falls, sports injury among others (Annegers and Coan, 2000; Klein et al., 2003; Lossius et al., 2005; Misra and Kalita, 2009). The involvement of these factors is not solely related to epilepsy, since the onset of CNS disorders show that they may be potentially involved in inducing or aiding the progression of the disorder (Gavett et al., 2010).

It was originally considered that epilepsy was solely a neuronal disease (Robel and Sontheimer, 2016). To improve the understanding of the disorder various approaches were undertaken with studies demonstrating this pathology does not encompass only neurons, but neural tissue as a whole. In the past decade, researchers have focused on the glial cells impact on mediating this pathology (Robel and Sontheimer, 2016). Also, the role of inflammatory mediators as modulators of CNS pathologies has been investigated with the role of cytokines having been extensively studied (De Simoni et al., 2000). Further research is required to better understand the biological mechanisms involved in this pathology, and discover more effective treatments.

8.2.1 Epileptogenesis

Epileptogenesis is the process by which a normal brain alters and develops epilepsy (Pal et al., 2001). In humans, this process is initiated via CNS injury and/or microbial infections. To induce this process in animals, scientists require to initiate status epilepticus (SE) defined as a continuous seizure period lasting over 5 minutes. It is induced by several factors including CNS injury and infections along with chemicals and/or specific protocols (Cherian and Thomas, 2009). The mechanisms involved in epileptogenesis result in neuronal death and a hyperexcitable neuronal population, which triggers the development of seizures (Pitkänen et al., 2002). Hyperexcitation occurs due to an excess depolarization of neurons resulting in an abnormal release of neurotransmitters and action potential discharge. Traditionally, studies concentrated on sodium and potassium pumps along with the importance of their concentration gradients that tend to provide a transmembrane potential of -60mV (Scharfman, 2007). Researchers still continue to study these pumps and studies have demonstrated that extracellular ion concentrations may be regulated by glia, with many experts considering that glia cells play a role alongside neurons in the regulation of epileptic activity (Vezzani et al., 2012).

However, the individual mechanisms involved in the overall epileptogenesis process are currently not entirely understood. The process of epileptogenesis includes several determined hallmarks, with the most notable being neuronal death, gliosis and re-wiring. These hallmarks are believed to result from the abnormalities previously mentioned (Pitkänen et al., 2002; Shapiro et al., 2008; Sloviter, 1999). Another factor that may potentially be involved is inflammation since increased molecular inflammatory markers have been observed (De Simoni et al., 2000). No drugs are currently being marketed which target and prevent epileptogenesis. However, it is considered to be a potential target due to it preventing the onset and progression of the disorder (Jacobs et al., 2009).

8.2.2 The Hippocampus and Temporal Lobe Epilepsy

The hippocampus is a major component of the brain that is involved in melding information from short-term memory to long-term memory along with having a vital role in spatial memory (Alonso et al., 2002; Broadbent et al., 2004). This structure is located in the medial temporal lobe and is considered a seizure-prone structure, since here the principal cells display the potential for epileptiform activity and seizures (Khrestchatisky et al., 1995). However, it is unknown if the cause of

epilepsy is the presence of hippocampal abnormalities or whether it is the cumulative effects of seizures that result in neuronal tissue damage (Norwood et al., 2010; Thom et al., 2005)**.**

One of the most frequent forms of epilepsy is Temporal lobe epilepsy (TLE) and it accounts for most of the recorded partial epilepsies (Wiebe, 2000). It is either familial or sporadic with onset mostly affecting children and teenagers. The sporadic form tends to be caused by head injury with studies associating it with an increase in the likelihood of developing TLE (Annegers and Coan, 2000). The use of magnetic resonance imaging (MRI) allows medical professionals to determine whether the cause of the sporadic form is lesion or non-lesion related (Téllez-Zenteno et al., 2010).

Most of the patients suffering from TLE show structural abnormalities in the hippocampus that are referred to as hippocampal sclerosis. This consists of selective and extensive neuronal loss and gliosis in the hippocampus, mostly in the cornus ammonis 1 (CA1) and dentate gyrus (DG) regions (Seress et al., 2009; Tan et al., 2015). Another feature includes the appearance of mossy fibre sprouting, defined as the synapse formation that mossy fibre axons of the DG region undergo, either with themselves or with other granule cells (synaptic reorganization). This process may cause a monosynaptic excitatory feedback although it is unknown whether this significantly decreases the threshold for seizure like events (Scharfman et al., 2003; Seress et al., 2009). This feature has been demonstrated in both animal models and human brain tissue from TLE patients. Thus, mossy fibre sprouting has been greatly studied to understand the hyperexcitability enhancing roles (Scharfman et al., 2003).

8.3 CURRENT ANTIEPILEPTIC DRUGS AND THEIR LIMITATIONS

This disorder is primarily treated with drugs, although in several cases surgical intervention may be required. Current drugs can only reduce and prevent seizures so are referred to as anti-epileptic drugs (AEDs) since they attempt to provide better seizure control. Thus, current available AEDs are mainly directed at preventing symptoms, since they only block seizures without curing the patient (Löscher, 2012). Also, these drugs are used to prevent convulsions caused by other diseases and infections (see further details on seizure causes in section 8.2). Therefore, they are often termed anti-convulsants (AC) instead of AED (Misra and Kalita, 2011).

These drugs tend to have several drawbacks since they are considered to cause side effects that vary from patient to patient. Individuals whom require AED treatments often report side effects that include amplification of fatigue, headaches and vomiting. One of the recent third generation AEDs is Eslicarbaepine (aptiom®), that is metabolized and converted into eslicarbazepine, which modulates the activity of voltage-gated sodium and calcium channels (Tambucci et al., 2016). Studies evaluating this drug concluded it was well tolerated with approximately between 20-30% having secondary effects including dizziness and fatigue. Approximately 10-15% of individuals halted medication since the side effects were too adverse (Holtkamp et al., 2016; Serrano-Castro et al., 2013). Epileptic individuals are required to take AEDs every day to control their seizures and this accumulation may lead to drug toxicity, which then plays a role in the adverse effects (Panayiotopoulos, 2005). Indeed, newer AEDs are still demonstrating side effects due to its accumulation being above the individual's tolerance (Avanzini, 2006).

Current AEDs are able to prevent seizures in most epileptic individuals, but approximately 24% of patients are resistant to epileptic drugs despite improvements in diagnosis and number of available AEDs (French, 2006). In these pharmaco-resistant individuals, medication does not aid in controlling seizures and they are referred to as having refractory epilepsy. New AEDs or those still in the research phase are aimed at having better efficacy (decreasing the number of individuals with refractory epilepsy) and less side effects in comparison to their predecessors.

8.4 GLIAL CELLS

Studies have provided evidence demonstrating the importance of glial cells in physiological and pathological conditions. Nowadays, it is considered that glial cells are important for the functional organization of the brain and aid neurons in brain development (Cerbai et al., 2012). Studies have discovered four types of glial cells in the CNS and these subsets include microglia, astrocytes, radial glial cells and oligodendrocytes (Zhu et al., 2009). It is considered that both microglia and astrocytes are the activated glia types in most CNS disorders and they have been studied along with neurons (neuron-astrocyte-microglia triad) to determine the mechanisms and processes that may occur (Sheridan and Murphy, 2013). Also, it is physiologically important that neurons in the injured brain are protected (Jeong et al., 2013). It is considered that this is accomplished by microglia and astrocytes with some involvement of other glial cell types (Szalay et al., 2016).

Astrocytes are located throughout the CNS surrounding the neurons both in physiological and pathological conditions (Agarwal and Bergles, 2014). They are considered to have a role in both the pre- and post-synaptic terminals through the release of gliotransmitters. Thus, astrocytes are considered a crucial element in the tripartite synapse and are involved in synaptic modulation under physiological conditions (Perea et al., 2009). Studies have demonstrated that astrocytes have a critical role in neuroinflammatory processes since they respond to cell stress and various stimuli that arise from CNS damage and infection. Also, they may have a role in pro-inflammatory pathways since they release growth factors (for tissue repair) and pro-inflammatory mediators such as cytokines (Lin et al., 2006; Nair et al., 2008). In terms of cellular morphology, at sites of injury or infection, these cells become activated which corresponds to cellular proliferation along with the observed extending and upregulated cellular processes that emerge from the central astrocyte cell body. Altogether, these features are often referred to as a glial scar that plays an important task in the roles previously mentioned (Shapiro et al., 2008).

The resident macrophage cells of the CNS are the microglia that sense and respond to either injury or infections (Lively and Schlichter, 2013). Normally, in the absence of danger or pathogenic signals the microglia roam the brain in a resting state, which involves monitoring the environment along with having the ability to oversee neuronal and astrocytic activity. In this state, microglia have a ramified morphology resembling the dendritic cells from the innate immune system with evidence suggesting they share similar cell pathways and functions (Nimmerjahn et al., 2005; Savage et al., 2012). Following microglia activation in response to CNS injury or proinflammatory stimuli, cell morphology rapidly changes to a primed state, which is characterised by shortened processes and a larger round cell body shape. Following priming, these cells become activated and this is referred to as an amoeboid activated state. While in this state and at sites of damage, microglia have the ability (as do dendritic cells) to release various inflammatory mediators, the most common and well known being the cytokines. Also, microglia cells have the ability to migrate through the parenchyma towards the lesion site in response to stimuli. This seems to occur due to the cellular movement via adhesion that are disintegrating at the rear and forming at front (Lively and Schlichter, 2013; Nimmerjahn et al., 2005). Also to aid in microglia being mobile they release enzymes that degrade the extracellular matrix aiding microglia in being mobile (Lively and Schlichter, 2013). Activated microglia move their shortened process and the whole cell body, while in the resting state microglia rely on their processes for movement (Avignone et al., 2015).

Like dendritic cells, activated microglia can undergo endocytic and phagocytic activity along with having the ability to serve as antigen-presenting cells (Cerbai et al., 2012; Mack et al., 2003). The beneficial effect of the phagocytic activity is the removal of cellular debris and toxic substances, along with the removal of host cells infected by pathogens, impeding their spread to other cells. In this way,

microglia provide a pro-survival ability to surrounding cells (Sierra et al., 2010). In terms of microglial cell number, studies have demonstrated a higher proliferation ability of these cells in response to stimuli, thus increasing the number of microglial cells that respond, also referred to as microgliosis (Hatori et al., 2002). It is considered that microglia have two phenotypes, being pro- and anti-inflammatory, releasing the main cytokines being interleukin-1β (IL-1β) and interleukin-10 (IL-10) respectively (Lobo-Silva et al., 2016). The IL-1β is explained further in section 8.5.2.1. The anti-inflammatory cytokine, IL-10, binds to IL-10 receptors and induces signalling cascades that result in reduced pro-inflammatory cytokines such as IL-1β (Kwong et al., 1998).

In terms of CNS disorders such as epilepsy, astrocytes may initiate and amplify the pro-inflammatory mechanisms with evidence obtained from both *in vitro* and *in vivo* models (Farina et al., 2007; Seifert et al., 2010). Thus, studies have been undertaken to understand their roles in CNS pathologies. Studies have demonstrated that, although both astrocytes and microglia may respond to inflammation, microglia are much faster at responding to stimuli (Block and Hong, 2005; Farrar et al., 2012). One of the general functions of glial cells is their role in modulating synaptic transmission (Barres, 2008; Kettenmann et al., 2011; Perry and O'Connor, 2010) and evidence suggests that alterations in these cells may allow them to have a functional role in hyperexcitability (Binder and Steinhäuser, 2006). Studies demonstrated that alterations in terms of neurotransmitters, inflammatory mediators and hyperxcitability, which is typical of epileptic disorders (Binder and Steinhäuser, 2006; Vezzani et al., 2008). Also, studies have demonstrated evidence of blood-brain barrier (BBB) disruption allowing the passage of peripheral immune cells that aid microglia in the progression of neurodegenerative disorders (Dantzer et al., 2008; Deleidi et al., 2015). Therefore, activation of glial cells is intimately associated with CNS disorders and further understanding of this association is nowadays considered mandatory (Barres, 2008).

8.5 NEUROINFLAMMATION

Inflammation is the non-specific immune response that occurs in reaction to any type of bodily injury, resulting in several signs, such as increased blood flow, swelling, redness and elevated cellular metabolism (Ferrero-Miliani et al., 2007). Both the brain and spinal cord are unique. They do not share the systemic inflammatory system, since they are constrained by the BBB and by the blood-spinal cord barrier (BSCB), respectively (Prinz and Priller, 2017). Moreover, the CNS contains its own immune cells, which are the microglia and astrocytes (see section 8.4), which ensure immune responses through the CNS (Aloisi, 1999).

Most CNS injuries result in activation of inflammatory pathways, which is important for responding to injury. In terms of acute injury it is currently widely understood that the inflammatory responses have two purposes: bactericidal action and repair (Mantovani et al., 2013). Bactericidal functions allow the protection of damaged tissue from infection, while repair functions are initiated to aid in the recovery of damaged tissue. However, brain injury is different. Firstly, in many cases, as internal lesions, the skull is intact and there is no occurrence of infections. Secondly, neurons lack the ability to be regenerated and once damaged they cannot be replaced. Even though, the latter is traditionally considered, studies have demonstrated the role of inflammation in the regeneration of neurons. Therefore, experts have been observing inflammatory functions to understand their significant roles in neuroinflammation and how they potentially protect damaged neurons (Jeong et al., 2013).

Inflammatory processes play a role in many neurodegenerative disorders such as Alzheimer and Parkinson's disease. In recent years it has been considered to play a role in epilepsy and to be a potential contributor for epilepsy onset and seizure progression (Vezzani, 2014). Studies have

demonstrated that neuroinflammation promotes increased neuronal excitability and lead to the secretion of pro-inflammatory cytokines within the CNS (see below in section 8.5.2).

8.5.1 Toll-like Receptors & High Mobility Group Box 1

Toll-like receptors (TLR) are a class of membrane receptor proteins which enable innate immune cells to recognise and respond to pathogens (Charrel-Dennis et al., 2008; Nociari et al., 2007) and host-derived molecules (such as fibronectin; Okamura et al., 2001). In the CNS, it has been demonstrated all cells express TLRs but each cell type displays its own subset and related signalling adaptor proteins. Also, the cell type that predominantly expresses these proteins are the microglia (TLR2, TLR4 and TLR9). Another important component of the inflammatory cascade is the High Mobility Group Box 1 (HMGB1). In the CNS it is expressed in various cell types including neurons and microglia. It is released extracellularly during events of sterile injury such as necrosis (Fang et al., 2012; Maroso et al., 2010).

Neural disorders have been shown to implicate TLRs while in 2010, Vezzani and colleagues showed TLR4 and HMGB-1 are implicated in generating seizures. In the same study they used an HMGB1-TLR4 inhibitor which resulted in seizure reduction by up to ~75% after about 2 hours of administration (Maroso et al., 2010). Recent studies believe TLR activation may play a role in inflammatory pathways involving the formation of a protein complex required for processing and activating caspases; this is explained further in section 8.6 (Grishman et al., 2012).

8.5.2 Cytokines

The main inflammatory regulators are the cytokines, small molecular proteins involved in the innate and adaptive immune response of the body (including the CNS). This class of proteins includes interferons and interleukins which are all secreted by pro-inflammatory cells. They act as signalling molecules via interacting with specific complementary receptors and cell pathways inducing a specific response relative to the target cell (Basu et al., 2002). In the CNS both the cytokines and their complementary receptors have considerably low expression levels and become upregulated upon an inflammatory stimuli (Utsuyama and Hirokawa, 2002). The following actions of the cytokines in response to a specific inflammatory insult can be either neurodegenerative or neuroprotective (Lobo-Silva et al., 2016). Further evidence suggests cytokines may modulate synaptic plasticity and reorganization (re-wiring), inducing changes in neuronal connections (Khairova et al., 2009).

Cytokines appear to be heavily implicated in epilepsy disorders, with studies demonstrating various changes associated with the overexpression of cytokines. Specifically, these involve synaptic dysfunction and excitotoxicity along with the cellular death of neurons. These occur in the epileptogenesis process and during seizures. It is considered that cytokines aid seizure induction with the seizure simultaneously contributing to increased cytokine expression and release from cells. Cytokines are involved in various types of programmed cell death that occur in epileptic disorders with neuronal death being a main feature of this disorder and is explained further in section 8.7. The synaptic dysfunction relies on cytokines decreasing the seizure threshold, thus altering the action of neurons and neurotransmitters. Nowadays, due to its pivotal role in epileptogenesis, neuroinflammation is a popular topic of study (Vezzani et al., 2012).

The main cytokines considered to influence both the onset and propagation of epilepsy are interleukin-1β (IL-1β), tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6). IL-1β and interleukin-18 (IL-18) are further described ahead (in sections 8.5.2.1 and 8.5.2.2), since the same proteolytic enzyme, caspase-1, processes them both. Also, these interleukins and caspase are involved in a form of programmed cell death called pyroptosis, which is explained in section 8.7 (Chen et al., 2014).

8.5.2.1 Interleukin-1β

The principal pro-inflammatory mediator involved in neurodegenerative diseases is IL-1β. All members of the IL-1 family are expressed within the CNS, with IL-1β being the most studied in various CNS pathologies. This popularity is due to it being the first cytokine released under pathological conditions along with its ability to upregulate expression and release of other cytokines (Srinivasan et al., 2004). This protein is synthesized as a low molecular weight (31 kDa) inactive precursor, called pro-interleukin-1β (pro-IL-1β) that undergoes processing via caspase-1. This caspase is activated by the multi protein complex (MPC), inflammasome (see section 8.6), from its synthesized form to its active form; it proteolytically cleaves pro-IL-1β to its active form, IL-1β (17 kDa). Following maturation, IL-1β can be released from the cell allowing it to act on a target cell (Martinon et al., 2002). Target cells express specific membrane receptors that are complementary to IL-1β, for example the Interleukin-1 receptor type I (IL1-RI). Following this interaction there is a simultaneous association of the receptor with the receptor accessory protein forming a complex (IL-1RAcP). This complex initiates an intracellular signal via the bound adaptor proteins, myeloid differentiation factor 88 (MyD88). Then, this signal triggers intracellular pathways that lead to the activation of several transcription factors including, nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs) among others (Liège et al., 2000; Palová-Jelínková et al., 2013; Ricote et al., 2004).

Another receptor of IL-1β is receptor Interleukin 1 receptor type 2 (IL-1RII) that has higher affinity for IL-1β in comparison to IL-1RII. The IL-1RII lacks a cytoplasmic domain so it does not trigger a signalling cascade when it binds to IL-1β. Thus, IL-1RII acts as an anti-inflammatory agent since the binding of IL-1β results in the inhibition of pro-inflammatory intracellular pathways (Ricote et al., 2004).

A variety of CNS cells may release IL-1β with the predominant producer being the microglia. Studies have demonstrated IL-1β production and maturation occurs following the pro-inflammatory stimulus (Kim et al., 2006). As mentioned before, there is an association between cytokine upregulation and seizure induction. One of the implicated cytokines is IL-1β, with evidence demonstrating the upregulation of this interleukin may decrease seizure threshold and increase neuronal excitotoxity (Dubé et al., 2005). For this reason, this interleukin is classified as a pro-convulsive molecule, with experts believing that inhibitors of its pro-inflammatory pathways may become successful anticonvulsants (Vezzani et al., 2008).

8.5.2.2 Interleukin-18

This interleukin is similar to IL-1β since they both belong to the IL-1 family and it also undergoes conversion from its pro-interleukin-18 (24 kDa) to the active form, IL-18 (17.2 kDa) via the proteolytic enzyme, caspase-1. In its matured form, it is released by the same cells that release IL-1β (for example, macrophages and dendritic cells) and interacts with its target cells at complementary receptors. IL-18 binds to interleukin-18 alpha receptor alpha (IL-18Rα) and beta (IL-18Rβ), with the latter having a higher affinity to bind IL-18 (Gerdes et al., 2002). Following the binding, IL-18 forms a complex similar to IL-1RAcP. The heterodimer formed allows the Toll-IL-1 receptors (TIR) to approximate since one TIR domain is bound to IL-18Rα and another domain to IL-18rβ. Following the approximation, MyD88 is recruited along with four Interleukin-1 receptor associated kinase-1 (IRAK) and tumour necrosis factor receptor associated factor-6 (TRAF-6). This results in the degradation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB). This protein inhibits the activation and subsequent release of NFκB, therefore when inhibited this action does not occur (Gerdes et al., 2002; Smeltz et al., 2001).

In relation to the CNS, studies have demonstrated there may be an association between IL-18 and CNS disorders such as Epilepsy. Evidence also demonstrates a role of this interleukin in neurodegeneration, which may aid in the onset of epilepsy (Anderson and Rodriguez, 2011; Felderhoff-Mueser et al., 2005).

8.6 THE INFLAMMASOMES

The large intracellular multi-protein complexes (MPC) called the inflammasomes, are involved in both the innate and adaptive immune response (Kobayashi, 2005). The term inflammasome refers to the activated oligomer when it includes either caspase-1 or caspase-5. In 2002, Tschopp and colleagues first described these complexes and demonstrated their involvement in intracellular inflammatory pathways. Their study demonstrated that inflammasomes trigger the activation of inflammatory caspases resulting in the processing of ProIL-1β to IL-1β (Martinon et al., 2002). Along with follow up studies, this MPC has been identified to be important for host component since are a vital component of pro-inflammatory cell pathways, important for host protection (Martinon et al., 2009).

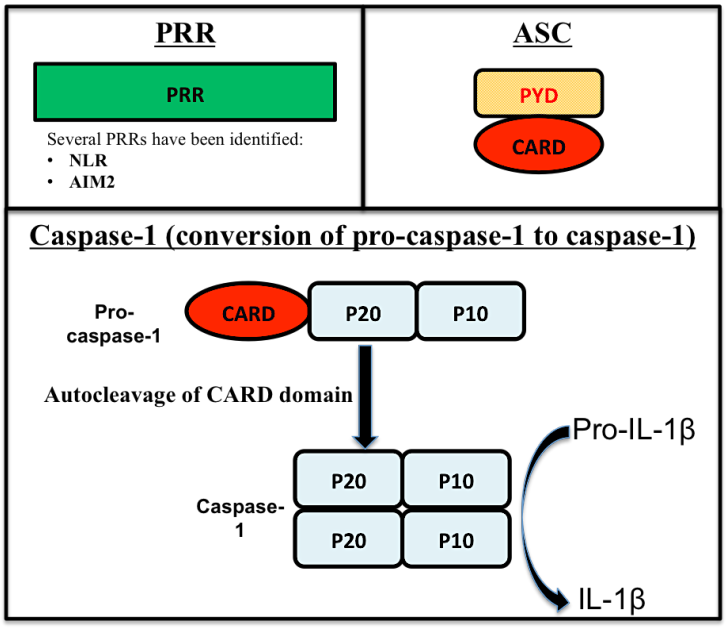

Figure 8.1 | The three main proteins (PRR, ASC, Pro-caspase-1) of the inflammasome. Studies have identified absent in melanoma 2 like receptor (AIM2) or one of the members of the nucleotide-binding oligomerization domain-like receptor (NLR) family of proteins to be the pattern recognition receptor (PRR) proteins of the inflammasome. Both can sense a variety of molecules that act as priming stimuli for inflammasome assembly. Apoptosis speck-like containing caspase recruitment domain protein attaches to an activated PRR via a PYD/PYD interaction. ASC interacts with procaspase-1 via a CARD/CARD interaction resulting in the autocleavage of the CARD domain of the procaspase producing a caspase-1 monomer that binds to a caspase-1 monomer forming an activated conformation. This activated caspase-1 can process pro-IL-1β to IL-1β and pro-IL-18 to IL-18. Other caspases are known to be an inflammasome component such as caspase-11. Original figures in Broz and Dixit, 2016; Gross et al., 2011; Jo et al., 2016.

The proteins that form this MPC are shown in **Figure 8.1** and include a pattern-recognition receptor (PRR) that acts as a sensing molecule, an apoptosis-related speck-like containing caspase recruitment domain protein (ASC) and a caspase (Sušjan et al., 2017). It has been determined that the inflammasome contains various PRR, ASC and caspase units, thus having a heterodimer structure (Harijith et al., 2014). Various PRRs have been identified and they define the type of inflammasome to be assembled along with the type of stimuli that initiates its activation. Also, these PRRs may contain a pyrin domain (PYD) that interacts with ASC via a PYD-PYD interaction, bridging both proteins

(Bae and Park, 2011). The ASC protein consists of a PYD and protein caspase recruitment domain (CARD), classified as death effector domains since they play a role resulting in caspase activation (Agostini et al., 2004). The CARD domain recruits an inactive procaspase to the inflammasome, with evidence suggesting it to be mostly pro-caspase-1. This is followed by cleavage and activation resulting in active caspase-1. This then cleaves and processes several pro-inflammatory cytokines allowing for their secretion (see section 8.5.2). Also, several PRRs already contain a CARD domain, therefore these are not dependent on the ASC for integrating with caspase-1. Further studies demonstrate that some PRR can lack a PYD while still having the ability to interact with ASC proteins (Broz et al., 2010; Chen et al., 2014; Martinon et al., 2002; Srinivasula et al., 2002). This interaction may have various roles in pro-inflammatory pathways and is explained further in 8.6.1.1.

Further knowledge about all inflammasome components is still required to determine their role in proinflammatory pathways.

8.6.1 Pattern-recognition-receptors of the inflammasomes

These PRR proteins tend to be either a nucleotide-binding oligomerization domain-like receptor (NLR; Howe et al., 2016) or the recently discovered inflammasome sensing molecule, absent in melanoma 2 like receptor (AIM2; Morrone et al., 2015). There are four family subsets of NLR that respond to different stimuli and are described further in section 8.7.1. The AIM2 receptor is a 343 amino acid protein that senses double-stranded deoxyribonucleic acids (dsDNA) via its HIN-200 domain and undergoes activation and caspase recruitment with ASC via a similar method to the NLR inflammasomes (Hornung et al., 2009). However, the process of oligomerization to form AIM2 inflammasomes potentially occurs via clustering at the binding sites of dsDNA unlike NLR receptors that have oligomerization domains (Morrone et al., 2015). Studies have demonstrated that different inflammasomes are expressed (in both low and high quantities) or not in different tissues and cells. Thus, demonstrating that each inflammasome may have a specific role and studies have been concentrating on each and their cell pathways (Yin et al., 2009). Below the NLR family is explained further since these are the most well studied PRRs.

8.6.1.1 Nucleotide-binding oligomerization domain-like receptor

Molecular and genetic studies have identified 23 human and 34 mouse NLRs along with defining the specific domains these tripartite structures contain and these are described in figure 8.2. They can be characterized into 4 sub families based on their known N-terminal domain, with each domain being in described in **Table 8.1**. These PRRs contain a common central nucleotide-binding and oligomerization (NACHT) domain that is usually flanked by a C-terminal leucine-rich repeat (LRR) domain and a PYD or CARD domain. Most NLRs contain a NACHT associated domain (NAD) located between the C-terminal of the NACHT and LRR domains. The NACHT domain is common among all NLRs and is vital for forming oligomeric structures; LRR tend to function in ligand sensing and autoregulation while CARD and PYD domains mediate downstream signal transduction (Martinon et al., 2001, Martinon et al., 2002). Also, studies using knockout mice demonstrated the NACHT domain is critical since inflammasomes are only active when in an oligomer configuration as demonstrated in **Figure 8.3**.

Table 8.1: The N-terminal domains of the 4 sub-families of the NLRs | The final letter of each sub-family is the commencing letter of the N-terminal domain (Dagenais et al., 2012).

NLR sub-family	N-terminal Domain
NLRA	Acidic transactivation domain
NLRB	Baculoviral inhibitory repeat domain
NLRC	Caspase-recruitment domain (CARD)
NLRP	Pyrin domain (PYD)

These platforms are considered to respond to different priming stimuli and are located in various cells and tissues. Early studies used transfected cell lines and/or knockout mice to identify the various stimuli for an inflammasome response. Several of the NLRs along with their respective stimuli are demonstrated in figure 8.2. Several NLRs contain CARD therefore they do not rely on interactions with ASC. The NACHT, LRR and PYD domains-containing protein 1 (NALP1), contains both a PYD and CARD; evidence suggests this protein can activate procaspase-1. Also, the NLR family CARD domain-containing protein 4 (NLRC4) is the only NLRC with the ability to form an inflammasome. It contains a CARD domain providing this NLR with a similar function. Both of these NLRs initiate pathways that are either dependent or independent of ASC especially since NLRP1 contains PYD that interacts with the PYD of the corresponding ASC. While, evidence suggests the ASC dependent pathway involving NLRC4 appears to involve ASC as a regulator, may mediate the integration of NLRC4 into an MPC (Broz et al., 2010; Case and Roy, 2011; Martinon et al., 2002),

The stimulus that these specific NLRs detect and elicit an effective response depends on the type of PRR since they may specifically sense pathogens, endogenous host danger signals or both. These are classified into pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) and most NLRs have the ability to sense one of these specifically as shown in **Figure 8.2** (Martinon et al., 2009).

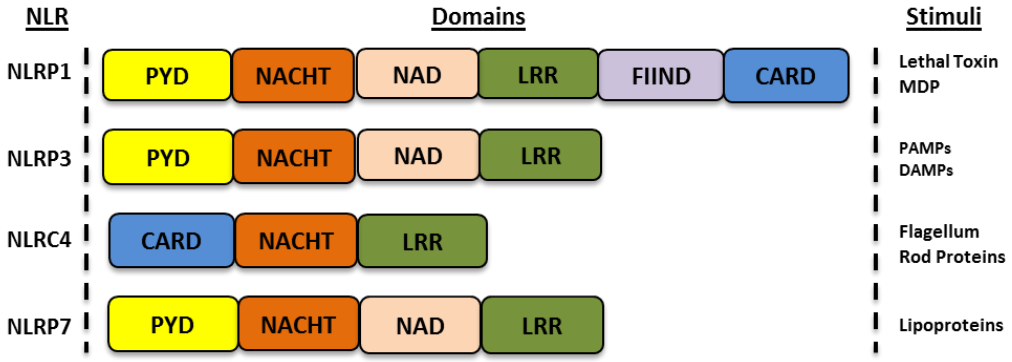

Figure 8.2 | The domains of various members of the NLR protein family. All NLR subtypes contain a NACHT domain, crucial for joining two of the same NLR subtype along with being one of the initial steps of inflammasome assembly. The subtypes with NLRP in their identification contain a PYD domain while those with NLRC contain a CARD domain. NLRP1 is unique since it contains both PYD and CARD; thus, NLRP1 can directly interact with CARD (via function to find domain, FIIND) of a pro-caspase (such as pro-caspase-1). Each NLR subtype respond to different stimuli, NLRP1 responds to lethal toxins and muramyl dipeptide (MDP); NLRP3 respond to pathogen associated mediated pathogens (PAMPs) and danger associated mediated pathogens (DAMPs) and this is explained further in section 8.6.2; NLRC4 responds to flagellum and rod proteins while NLRP7 responds to lipoproteins. Original figures in Eitel et al., 2011; Martinon et al., 2009.

8.6.2 The NLRP3/NALP3 Inflammasome

The most well studied inflammasome is the NLRP3 inflammasome and the selected focus of this present study. The PRR of this inflammasome is the NACHT, LRR and PYD domains-containing protein 3 (NALP3) and is encoded by the NLRP3 gene. Literature tends to refer to this protein either by using the synonym given to the gene (NLRP3) or referring to it as cryopyrin (Martinon et al., 2002). This PRR belongs to the NLRP inflammasome family since it contains a NACHT domain along with the N-terminal domain being PYD. Although various inflammasomes have been identified, NLRP3 inflammasome has the ability to detect and respond to various signalling stimuli including pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs), unlike most inflammasomes that only respond to one type (Kingsbury et al., 2011; Martinon et al., 2009). Several of these PAMPs and DAMPs, which prime NLRP3 activation, are listed in **Table 8.2** below.

Table 8.2 | Priming stimuli for NLRP3 activation. This table includes some pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) that act as stimuli for NLRP3 (Kingsbury et al., 2011; Martinon et al., 2009).

PAMPs	DAMPs
Bacterial RNA	B-amyloid
E.Coli LPS	Monosodium dehydrate crystals
MDP	Glucose
α-toxin	Cholesterol

This priming and the undergoing response result in transcriptional initiation and post-translational modifications of NLRP3 allowing for the activation of this intracellular receptor. Studies have demonstrated various NLRP3 stimuli require cellular potassium release, with evidence demonstrating that a low potassium ion (K^+) concentration is sufficient to activate NLRP3. Various factors that NLRP3 respond to (bacterial pore forming toxins) may form pores in the cellular membranes allowing for extracellular K^+ release. Therefore, reduction in intracellular K^+ levels may be the convergence point for most NLRP3 stimuli (Kaushik et al., 2012; Petrilli et al., 2007). However, it is unknown if this directly activates NLRP3 or if other cellular events may be occurring resulting in NLRP3 activation. Evidence suggests that other ions are involved in NLRP3 activation, such as calcium which activates NLRP3 via G-protein coupled calcium sensing receptors (Rossol et al., 2012). Another feature of NLRP3 activation is its requirement for the catalytic domains of NIMA-related kinase 7 (NEK7) that acts on the intracellular receptor downstream of K^+ extracellular release. NEK7 binds to and controls the oligomerisation process of NLRP3, crucial for NLRP3 inflammasome assembly (He et al., 2016).

The stimuli previously mentioned cause the proteins to undergo oligomerisation and recruit caspases via the domain, CARD, of the ASC protein, and the cysteine protease, procaspase-1, leading to both the autocatalysis and activation of caspase-1 (see NLRP3 inflammasome structure in **Figure 8.3**; Bae and Park, 2011). Active caspase-1 is involved in the maturation and processing of pro-IL-1β and pro-IL-18, producing the active IL-1β and IL-18, respectively (Felderhoff-Mueser et al., 2005; Martinon et al., 2002). As mentioned before, both interleukins are involved in a form of cellular death referred to as pyroptosis and this is explained further in section 7.11.

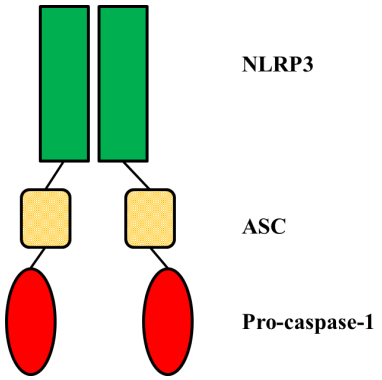

Figure 8.3 | The structure of the NLRP3 inflammasome. For the NLRP3 inflammasome to be assembled and active, evidence suggests it requires to oligomerise initially at the NACHT domain of the NLRP3 protein with that of another. Following activation of NLRP3 and oligomerization, ASC is recruited that allows for interactions with pro-caspase-1. This allows pro-caspase-1 processing that forms caspase-1, triggering IL-1β processing. Original figure in Martinon et al., 2009.

Studies have demonstrated some unique features of this inflammasome, such as its release following activation (similar to HMGB1 release), with the conformation of the structures located extracellularly appearing to be oligomer particulates (Baroja-Mazo et al., 2014; Scaffidi et al., 2002).

8.6.3 Inflammasomes of the CNS and its disorders

In terms of expression sites within the body, studies have demonstrated their expression in most parts including the CNS, lungs and kidney. In the CNS, various cell types express inflammasomes with studies investigating their locations of expression and their functions along with involvement in certain pathways. Astrocytes have weak NLRP3 expression and lack the inflammasome component, ASC (Gustin et al., 2015). Researchers consider that this low amount of ASC may prevent inflammasome formation preventing activation of caspase-1. In human astrocytes there is evidence demonstrating a novel NLRP2 inflammasome expression (Minkiewicz et al., 2013), while in rat astrocytes no expression was detected (Gustin et al., 2015).

Neurons express inflammasome components to a certain degree with cortical neurons expressing various PRRs including NLRP1, NLRP2 and Aim2. However, an assessment of their function is still unclear and this area requires further investigation. Microglia express NLRP3 and react to various typical NLRP3 activators and evidence shows they share several features with peripheral macrophages.

In an epilepsy context, evidences suggests NLRP3 is crucial for brain inflammation which contributes to epileptogenesis onset/development, with epileptogenesis in turn increasing brain inflammation mediators (Meng et al., 2014; Savage et al., 2012). Using the kindling induced SE model, SE rats were found to have an upregulation of NLRP3 that when exposed to NLRP3 short interfering ribonucleic acids (siRNA) a decreased protein expression of both caspase-1 and IL-1β was observed. This decreased expression was associated with decreased hippocampal neuronal loss suggesting NLRP3 may represent a potential target for the treatment of epileptogenesis (Meng et al., 2014).

8.6.4 Inflammasome inhibitors

Studies with mice deficient in ASC and NALP3 have demonstrated decreased IL-1β processing and release (Mariathasan et al., 2004; Sutterwala et al., 2006). This represents a potential target for manipulating the innate and adaptive immune response that may prove beneficial in several disorders where the inflammasome is implicated. Nowadays, targeting the inflammasome is considered to be a viable option for treating various inflammatory related diseases (Duewell et al., 2010; Lamkanfi et al., 2009; Youm et al., 2015). Various compounds are currently under investigation including β-hydroxybutyrate (a ketone body) and diarylsulfonylurea-containing compounds (Perregaux et al.,

2001; Youm et al., 2015). Also, microRNA (miRNA) specifically miR-223, have been demonstrated to limit the inflammatory capacity in developing myeloid cells (Haneklaus et al., 2012).

The referred compounds specifically target the NLRP3 inflammasome, potentially ceasing activation and release of IL-18 and IL-1β. Targeting the NLRP3 inflammasome with compounds provides a potential advantage over IL-1β suppressive therapies and new innovative therapies targeting other inflammatory cascade proteins including HMGB-1 and TLRs. Using these compounds, it would potentially allow IL-1β activation by other inflammasomes (NLRP1, NLRP7, etc.) providing a potential immune response against infections and pathologies (Perregaux et al., 2001; Youm et al., 2015). In other words, it would allow the immune system to still function to protect the host since several inflammasomes formed from other PRRs can still activate caspases (Coll et al., 2015).

8.6.4.1 MCC950

A recently studied potential inflammasome inhibitor was the composite, N-[[(1,2,3,5,6,7-hexahydro-s-indacen-4-yl)amino]carbonyl]-4-(1-hydroxy-1-methylethyl)-2-furansulfonamide, a diarylsulfonylurea-containing compound identified as MCC950 (Coll et al., 2015). As previously mentioned, these compounds are classified as interleukin-1 post-translational processing inhibitors and were later classified as cytokine release inhibitory drugs (Perregaux et al., 2001). Evidence suggests successful inflammasome assembly is inhibited by MCC950 since it prevents oligomerization from occurring that is fundamental for the processing of IL-1β (Coll et al., 2015).

This compound has been used in a wide range of pathology models, with studies demonstrating that it successfully inhibits the activation of the NLRP3 inflammasome (Lamkanfi et al., 2009). Evidence suggests that this compound prevents processing of IL-1β secretion induced by PAMPs and DAMPs (Coll et al., 2015; Lamkanfi et al., 2009). This action is demonstrated to prove advantageous, since it prevents the progression of various disorders including autoimmune disorders. Also, evidence suggests it does not inhibit other inflammasome types such as NLRC4, since cytokines that are modulated by this inflammasome are quantifiably unaltered in the presence of MCC950 (Coll et al., 2015). Thus, MCC950 has demonstrated to have a potential clinical use. However, further studies are required to understand the inhibitory mechanisms of these compounds, whereas clinical studies are needed to understand its potential use in humans (Levy et al., 2015).

8.7 NEURONAL DEATH

The association between neuronal death and seizures along with the role of seizure-induced neuronal death in epileptogenesis is an area that is heavily evaluated by neuroscientists. In animal models, SE has demonstrated its ability to induce neuronal death that is associated with commencing and aiding the development of the disorder including seizure induction. Thus, neuronal death has been proposed to be an integral part of epileptogenesis in SE models. However, studies focusing on SE in immature brains have evidence involving neuronal death not being a requirement for epileptogenesis (Baram et al., 2011)

Neuronal damage is attributed to excitotoxicity, the latter occurs by an increase in the amount of glutamate release and subsequent binding at excitatory synapses, since there is an over expression of glutamate receptors resulting in unordinary binding (Mou et al., 2014). This event is capable of provoking cell death by apoptosis, necrosis and pyroptosis (explained in sections 8.7.1 and 8.7.2) that may occur in neurons and various CNS cell types during epilepsy (Dingledine et al., 2014).

8.7.1 Necrosis and Apoptosis

The main difference between necrosis and apoptosis is that necrosis inflicts damage to the surrounding cells, while apoptosis does not (see **Figure 8.4**). Apoptosis is a form of programmed cell death and is initiated via stress signals that result in caspase-3 activation and cytochrome-c release. The cells bleb and break into several apoptotic bodies that contain organelles and cause no inflammation. Necrosis is a form of cell death that is caspase-independent and is induced by external factors such as infections. In this case, the cell blebs with the cellular contents released from the cell when it ruptures. The contents induce inflammation thus damaging surrounding cells (Fehsel et al., 2003). Evidence suggests both of these forms of cell death occur in epilepsy therefore are of a particular interest (Niquet et al., 2012).

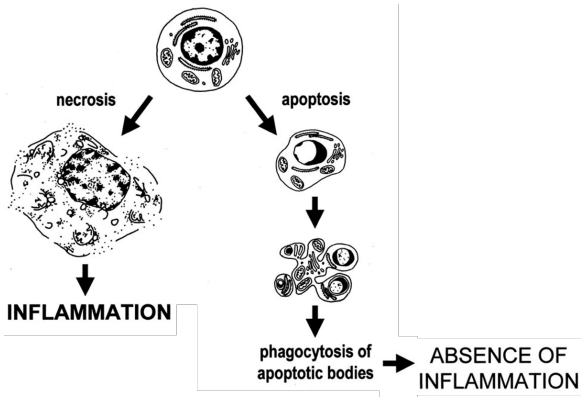

Figure 8.4 | Mechanisms of necrosis and apoptosis. Necrosis begins with the swelling of both the cytoplasm and mitochondria. Also, it is characterized by plasma membrane damage along with loss of cytoplasmic contents into the extracellular space, which leads to a significant inflammatory response. The cell undergoes complete lysis. Apoptosis is regarded as programmed cell death and is considered necessary for maintaining a homeostatic balance in the organism. This process is described by shrinking cells without the loss of plasma membrane integrity. Apoptotic cells are also characterized by condensation of nucleus (with DNA breaking) and formation of membrane bound vesicles (apoptotic bodies), where cellular contents are packaged. Subsequently, the apoptotic bodies are phagocytosed resulting in no inflammatory reactions subsequently occurring. Adapted from Fehsel et al., 2003.

8.7.2 Pyroptosis

Pyroptosis is a form of cell death that requires an inflammatory response and occurs mostly upon infection with intracellular pathogenic stimuli. The main mechanism that specifically differs pyroptosis from other forms of cell death is caspase-1 involvement. The inflammasome is considered to be associated with pyroptosis, since the inflammasome complex processes and activates the inactive pro-caspase-1 form (Chen et al., 2016).

Caspase-1 also has a role in the processing of gasdermin D (He et al., 2015) with caspase-11 also been shown to undergo the same process (Kayagaki et al., 2015). Gasdermin D undergoes cleavage by the previously mentioned caspases producing an N-terminal fragment, which has been demonstrated to form pores in the plasma membrane that is fundamental for initiating pyroptosis (He et al., 2015; Liu et al., 2016).

Pyroptosis was initially discovered by inducing cell death in macrophages via infection with *Salmonella* and *Shigella* strains of bacteria that resulted in cell death induction that differed from known forms of cell death along with a pro-inflammatory effect. Pyroptosis provides protection against infection due to the death of the infecting agent and is an efficient manner of clearing pathogens such as bacteria (Miao et al., 2010). However, pyroptosis may have a negative effect and

contribute to several diseases by aiding in T-cell depletion, as seen in human influenza infected hosts (Doitsh et al., 2014).

Various features have been discovered for this form of cell death. Loss of plasma membrane integrity and extracellular release of the cytoplasmic content are features shared with necrosis, but not with apoptosis. As is typical in cell death, the membrane swells as cellular size increases with the nucleus undergoing rounding and condensation. The nuclear integrity is maintained, unlike in apoptosis, while the undergoing DNA fragmentation is observed, as is in apoptosis and necrosis. As previously mentioned, it induces inflammation as necrosis does, but it has a more direct effect and less damaging effects on neighbouring cells (Liu et al., 2016). In the CNS, various cell types (neurons, microglia, etc.) can undergo pyroptosis, with the only difference among these cell types being the inflammasome that initiates the process. This is so since different cell types assemble different inflammasomes (Kim et al., 2015; Tan et al., 2014).

In relation to CNS disorders, pyroptosis is involved in various pathologies including Alzheimer's disease (Tan et al., 2014) and CNS microbial infections (Kaushik et al., 2012). In terms of epilepsy, studies have demonstrated a link between this disorder and inflammasome-induced cell death, along with subsequent evidence of pyroptosis occurring in neurons of epileptic neural tissue. Recent studies have shown that NLRP1 or caspase-1 silencing in the amygdala kindling-induced rat model was able to reduce neuronal pyroptosis (Tan et al., 2015).

8.8 EXPERIMENTAL MODELS OF EPILEPSY

The understanding of epilepsy requires the usage of appropriate animal models, since clinical studies with humans provide insufficient information on the mechanisms that may occur. The use of animal models has provided an enormous contribution to defining the cellular, molecular and electrophysiological mechanisms of epileptogenesis. This is accomplished by their ability to mimic various aspects of the disease and provide a method to visualize the mechanisms involved in hyperexcitation and seizure generation. Also, they are employed in discovering and testing potential AEDs in halting recurring seizures (Carter et al., 2011).

Rodent models (both rats and mice) are widely used to assess epilepsy via *in vivo* and *in vitro* methods (Engel and Schwartzkroin, 2006). The *in vivo* model tend to require a large number of animals due to the high mortality, along with requiring more extensive upkeep, involving high cost and time (Albus et al., 2008; Löscher, 2002). The *in vitro* model provides analysis of epilepsy while decreasing the need of the lab personnel having experience in animal handling together with the ease of cultures in comparison to using animals (Löscher, 2002). Unlike *in vivo* models, the *in vitro* models of epilepsy do not allow behaviour and motor assessment of clinical seizures (Heinemann et al., 2006).

The most utilised *in vitro* preparation are the acute brain slices, which is popular due to the vast experiments that can be undertaken using these slices (Heinemann et al., 2006). Also, these preparations preserve the three-dimensional architecture and local environment of brain cells, including neurons, astrocytes and microglia, as well as the neuronal connectivity and the complex glial-neuronal interactions (Humpel, 2015; Noraberg et al., 2005). However, acute slices have a short viability since they only last a few hours, which limits electrophysiology studies and prevents the evaluation of long-term effects (Gähwiler et al., 1997). In comparison, the use of organotypic brain slices is considered to allow long-term maintenance and manipulation via various means including pharmacological agents (Albus et al., 2008; Gähwiler et al., 1997; Heinemann et al., 2006). These can be added directly to acute or organotypic slices, without interference from the rest of the body systems

and researchers do not require to concern themselves with limitations that may occur with passage via the BBB (Baraban, 2007; Wahab et al., 2010).

8.9 ORGANOTYPIC HIPPOCAMPAL SLICES CULTURE INVOLVED IN EPILEPTIFORM MODELS

The organotypic hippocampal slice culture (OHSC) is a preparation that is both useful and advantageous for the researcher in terms of studying neural tissue physiology and the alterations that occur under pathological conditions. These preparations have proved fundamental in the study of neuronal network dysfunctions due to their applications in electrophysiological, molecular and pharmacologic studies (Staal et al., 2011).

In these preparations slices are placed in a culture dish, or insert, in controlled conditions, it is thus termed *in vitro* cultures. However, since slices are isolated from the rest of an intact organism, while having the ability to be studied they are often called *ex vivo* cultures (Gähwiler et al., 1997; Stoppini et al., 1991). This system allows to evaluate the various underlying processes and mechanisms since it retains the complex architecture, properties and networks as the ones seen *in vivo*. In terms of survival and maintenance, they can be maintained in growth medium for several weeks and manipulated as is appropriate for the experiment by the researcher in a simple and effective manner (Heinemann et al., 2006).

Generally, the OHSC slices contain the hippocampus and cortex regions being obtained from post-natal (PN) rodents, specifically 6-7 days old pups (Opitz-Araya and Barria, 2011). At this age the hippocampus is established with the brain itself being at a degree of development allowing the brain architecture to be observable under a microscope (Engel and Schwartzkroin, 2006). Also, studies describe these slices are able to survive for several weeks in an incubator along with growth medium containing supplements for optimal survivability (Heinemann et al., 2006).

However, the physiology of neural tissues from young rodents is different to that of older ones since the brain and its processes are fully developed. Thus, slices obtained from pups does not resemble several of the features seen in adult rodents but they develop and mature similarly to the *in vivo* counterparts (De Simoni et al., 2003). However, the use of older mice proves to be fundamental for several research projects especially those that encompass pathologies that tend to have a higher occurrence in ageing. Indeed, the use of older animals (P50), instead of younger or neonatal pups, for preparing OHSC has been studied. Evidence suggests that tissue from neonatal mice survives longer, but has higher levels of astrocytic activation (Staal et al., 2011).

The procedure for the preparation of organotypic slices has several important features in terms of the slices physiology. Following preparation of the slices, evidence demonstrates robust degeneration of neurons up to 6 *days in vitro* (DIV), with a low degree of neuronal death occurring up to 28 DIV (Pozzo Miller et al., 1994). Additionally, the slicing process is associated with the reorganization of neural processes that is similar to the features observed in both chronic epileptic rats (Mello et al., 1993) and epileptic human patients (Franck et al., 1995). Also, evidence demonstrates that organotypic slices undergo development of mossy fibre sprouting and recurrent excitatory connectivity (features of TLE) along with the reorganization of the CA1 region (Dudek and Sutula, 2007). As a consequence of these alterations, excitatory activity progressively increases over time in culture (Bausch and McNamara, 2000).

However, these features are insufficient for the onset of spontaneous epileptiform activity and pharmacological induction, such as kainic acid, has been reported (Holopainen, 2005). A model was published where the onset and development of spontaneous epileptiform activity occurs without any pharmacological induction (Dyhrfjeld-Johnsen et al., 2010). A similar model was developed at the

Instituto de Medicina Molecular (Magalhães et al., 2017, in preparation) and has been previously studied. This study demonstrated that organotypic slices maintained in an artificial growth medium, without serum and supplemented with B-27, displays recurrent spontaneous epileptiform discharges together with many inflammatory features associated with *in vivo* epilepsy. Several inflammatory players, such as neuronal death, gliosis and up-regulation of pro-inflammatory cytokines were observed (Magalhães et al., 2017; in preparation). Therefore, this model may be a tool to evaluate the interplay between epilepsy and inflammation, as well as to screen potential drug candidates to reduce/halt epileptogenesis.

9 AIMS

The search for novel AEDs has been considerably directed at the use of anti-inflammatory agents. Targeting inflammatory pathways, is regarded as being potentially important for halting this disorder due to previous experimental studies demonstrating crosstalk between neuroinflammation and epileptogenesis. Thus, research studying the affects of anti-inflammatory drugs on epilepsy are imperative.

The aim of this study was to determine if inhibiting the NLRP3 inflammasome using a diarylsulfonylurea-containing compound, named MCC950, in an *ex vivo* model of epileptogenesis had any effect on neuronal death, glial activation and epileptiform activity.

Several topics were evaluated throughout culture time,

- Cell death
- Astrocytes and microglia activation
- Electrophysiological recordings of spontaneous epileptiform activity

10.1 ANIMALS

Pregnant female Sprague-Dawley (SD) rats were acquired from Charles Rover (Barcelona, Spain). All experimental procedures were undertaken in accordance with current Portuguese Laws and the European Union Directive (2010/63/EY) on the protection of animals destined for experimental and other scientific purposes. All efforts were made to minimize animal suffering and to use only the minimal number of animals necessary for the required experiments.

10.2 PREPARATION OF ORGANOTYPIC HIPPOCAMPAL SLICE CULTURE

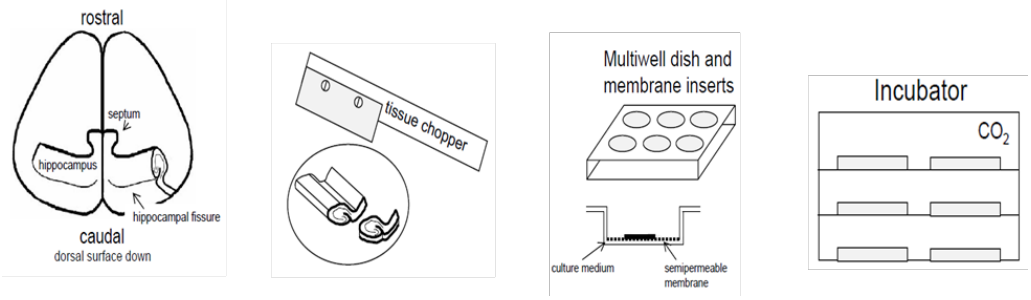

Figure 10.1 | Preparation of hippocampal slice cultures. The cerebral hemispheres were separated and sliced transversely at 350 µm using a Mcllwain tissue chopper. The combined hippocampal-entorhinal cortex slices were placed onto porous (0.4µm) inserts, which were transferred to a six-well culture trays and placed in an incubator at 37°C with 5% CO_2 and 95% atmospheric air for the following 1 to 3 weeks.

The OHSC were prepared from 6 to 7 days post-natal Sprague-Dawley rats, according to the interface culture method presented by Stoppini and colleagues (Stoppini et al., 1991). Following decapitation, heads were placed in cold Grey's Balanced Salt Solution (GBSS) (Biological Industries, Kibbutz Beit Haemek, Israel) with 25mM glucose solution (Sigma Aldrich, MO, USA). Brains were quickly removed and combined entorhinal cortex (EC), perirhinal cortex (PC) and hippocampus, were rapidly dissected out and sliced transversely at 350µM using a Mcllwain tissue chopper, as shown in Figure 10.1. Five slices were placed onto porous (0.4µM) insert membranes (PICM 03050, Milipore, Bedford, MA), which were transferred to six-well culture trays (Corning Costar, Corning, NY). Each of the wells contained 1mL of culture medium composed of 50% Opti-MEM solution, 25% Hank's balanced salt solution (HBSS), 25% heat-inactivated horse serum (HS) (all from Invitrogen, Paisley, UK), 25mM D-glucose, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Sigma). Slices were maintained in an incubator at 37°C with 5% CO_2 and 95% atmospheric air for the following 1 to 3 weeks as demonstrated in **Figure 10.1**.

Slices were randomly divided into two groups, which undertook different culture conditions. Those designated control slices (CTR) were maintained in a serum-based Opti-MEM medium, with medium renewal twice a week, since these did not develop spontaneous epileptiform activity (Magalhaes et al., 2017; in preparation). Those designated epileptic-like slices (EL), which spontaneously develop an epileptic phenotype were changed at 3 days *in vitro* (DIV) to a chemically defined serum-free based medium, Neurobasal A (NBA) (Invitrogen), supplemented with 2% B27 (contains several hormones, fatty acids, free radical scavengers, etc.) (Invitrogen), L-glutamine (1mM) (Invitrogen), penicillin (100 units/mL) and streptomycin (100µg/mL), and decreasing horse-serum concentrations (15%, 10% and

5%), until a serum-free condition was reached at 9DIV. Since several amino acids are rapidly consumed within 2 days and glutamine spontaneously degrades, in EL slices the medium was renewed every second day, for two weeks (14 DIV). All experimental assays were carried at 7 and/or 14 DIV.

10.3 COMPOUND

The NLRP3 inflammasome inhibitor, MCC950, was purchased from Cayman Chemical Company (Michigan, USA) and arrived as a solid. The provided product information stated the compound could be dissolved in culture medium, salt solution or DMSO. The selected solution was therefore the vehicle. The compound was diluted in DMSO, since it could be stored at -20^{o}C for the duration of the recommended storage period, to the stock concentration of 4mg/mL. The working concentration of MCC950 was 4µg/mL (10µM).

10.4 PHARMACOLOGICAL APPROACHES

For this study, organotypic slices from both CTR and EL slices were used. Only 7 and 14 DIV slices from the EL condition (obtained via the maintenance protocol stated in section 10.2) were incubated with MCC950 for a 6 hour (h) period.

EL slices from the same time points were exposed to further conditions including a selected vehicle solution being 0.1% dimethyl sulfoxide (DMSO) of which is the same solvent and concentration present in the condition using MCC950. DMSO is considered to have minimal effects on experiments when utilised in low concentrations. Studies have also demonstrated it to be an unspecific NLRP3 inhibitor at a concentration above 0.5% (Ahn et al., 2014). Therefore, samples from EL slices (no DMSO nor MCC950) were used to ascertain if any effects occur due to DMSO along with allowing the specific NLRP3 effect to be determined. The conditions used are stated below in table 10.1. All slices were removed and used in the experiments mentioned below following 6h exposure to vehicle or vehicle and compound.

10.5 WESTERN BLOT ASSAY

To ascertain protein expression, the western blot assay was utilized to separate and identify the proteins present in culture lysates. This assay separates proteins placed in an SDS-Polyacrylamide gel surrounded in buffer that allows a positive charge to pass through a buffer for the proteins to move. Both gel and buffer contain Sodium Dodecyl Sulfate (SDS) that ensures that all the proteins have the same negative charge allowing them to migrate according to their molecular weight. This technique is thus based on the molecular size, since heavy proteins have a larger molecular size and tend to migrate less within the gel compared to the lower molecular weight proteins. Proteins are first separated from the initial lysate and then are transferred to a membrane where they can be identified with specific antibodies.

10.5.1 Tissue lysates

The hippocampi were dissected from 4-5 slices and tissue lysis was performed in 150µL of RIPA (Ristocetin Induced Platelet Agglutination) buffer containing 50mM Tris pH 8.0, 1mM EDTA (Ethkenediamine Tetraacetic Acid), 150mM NaCl, 1% NP40 substitute (Nonyl phenoxlpoylethanol, Fluka Biochemika, Switzerland), 1% SDS and 10% glycerol.

Due to the cell disruption that occurs during the protein extraction process, protein degradation is prone to occur via facilitation by the endogenous proteases released by lysed cells. Therefore, inhibition of proteases was ensured by the addition of a protease inhibitor cocktail (Complete Mini-EDTA free, Roche, Germany) and 1mM PMSF (phenylmethylsulfonyl fluoride) to the lysis buffer.

Following cell lysis, cell suspension was left shaking for a period of 15 min at 4°C followed by removal of the insolubilized fraction via centrifugation, 11000g for 10 minutes at 4°C. Finally, the supernatant was collected into a new eppendorf tube and stored at -20°C, until further use.

10.5.2 Protein Quantification

Total protein was quantified using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The standard curve was performed with Bovine Serum Albumin (BSA), with 10µL of both standard and samples (the latter being diluted 10x) placed in 96-well plates (Thermo Fisher Scientific, USA). The plate was covered to prevent light exposure and left on a tilter for 15 minutes. Following the allotted time, bubbles were removed with a syringe needle, the plate was placed in Infinite M200 (Tecan, Switzerland) and the absorption was measured at 750 nm.

10.5.3 SDS-PAGE and Western Blot Transfer

Before samples were loaded onto the gel, they were boiled at 95°C for 10 minutes to allow denaturation of higher order structures. Samples (45µg total protein/well) and protein size marker (Protein Marker II, NZYTech) were run on a standard 12% SDS-PAGE gel at 120 volts. The proteins were electrotransferred onto a PVDF membrane (Immun-Blot® PVDF Membranes for Protein Blotting, Bio-Rad, California, USA) at a constant current of 300 Amps for 1h30. To verify if protein bands had been transferred, complete transfer of the protein marker was observed along with Ponceau staining to view protein band lanes of the samples.

Table 10.1 | Primary antibodies used for Western Blot. The host animal, manufacture and the working dilution are indicated. GFAP, Glial Fibrillary Acidic Protein; Iba1, Ionized Calcium Binding Protein – 1; NLRP3, NACHT, LRR and PYD domains-containing protein 3.

Antibody	Host	Manufacture	Working Dilution
αII-Spectrin	Mouse	Santa Cruz Biotech.	1:500
GAPDH	Mouse	Santa Cruz Biotech.	1:1000
GFAP	Rabbit	Sigma Aldrich	1:5000
Iba1	Goat	Abcam	1:1000
NLRP3 Inflammasome	Rabbit	NOVUS Technologies	1:300

Membranes were then blocked for 1 hour in TBS-T (200mM Tris pH7.5, 1.5M NaCl, 0.1% Tween-20) supplemented with either 3% BSA or 5% powder milk at room temperature (RT). Subsequently, membranes were probed with the primary antibodies (Abs) mentioned in **Table 10.1**, all diluted in TBS-T with either 3% BSA or 5% powder milk, overnight at 4°C on a rotating shaker. Finally, membranes were incubated for one hour at RT, with donkey anti-goat, donkey anti-mouse (1:10000, Santa Cruz Biotechnology) or goat anti-rabbit (1:10000, Bio-Rad) secondary antibody linked to horseradish peroxidase (HRP). Following probing at either stage, the membranes were washed in TBS-T for 30 minutes to remove any nonspecific attached antibodies thus reducing unnecessary noise during signal development. Development of signal intensity was undertaken using enhanced chemiluminescence substrate (Western Lightening® Plus-ECL; PerkinElmer, Massachusetts, USA). Signal was detected using ChemiDoc™ MP Imaging System (Bio-Rad) with an appropriate exposure time to suit the proteins being observed.

The relative expression of the protein bands was accomplished via selecting the image at a time before signal saturation had occurred and quantitatively analysing the signal intensity with software ImageJ 1.44b (NIH). All protein signal intensities were standardized to a housekeeping protein, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

10.6 IMMUNOHISTOCHEMISTRY & IMMUNOFLUORESCENCE MICROSCOPY

The ability of Abs binding specifically to complementary antigens (proteins) allows for the process of immunohistochemistry that involves detecting these antigens via the bound antibody. This study uses fluorescent reporter molecules attached to the secondary Ab, which are complementary to the primary Ab involved in the antigen-Ab interaction.

This method is used in immunofluorescence (IF) microscopy with the secondary antibodies coupled to fluorophores. These, like other fluorescent compounds, can absorb high intensity light of specific wavelength leading to the excitation of the fluorescent molecule resulting in part of the energy being emitted at a longer wavelength. These secondary proteins tend to be unspecific since they bind to primary antibodies of a host (host being the species such as rabbit, goat, etc.). This still allows multicolour immunoenzyme staining for different antigens since the complementary secondary antibodies can have a range of different fluorophores with different absorbance and emission features.

Using such method requires the user to take several factors into consideration. Tissues or cells to be stained, if in a degraded state, may prevent successful binding to complementary primary antibodies. Also, proper permeabilization of tissues and cells is mandatory and the antibodies concentration have to be optimal to avoid poor quality images. Furthermore, since fluorophores are light sensitive its photobleaching, i.e. inability to fluoresce, was prevented by keeping the samples protected from light after incubation with the fluorophore-coupled secondary antibody.

10.6.1 Slice fixation

Culture medium was removed from the culture tray and 1mL of 4% paraformaldehyde (PFA) in phosphate buffer solution (PBS) was added to each of the wells of the culture tray. Slices were allowed to fixate for a period of 1 hour at RT and the PFA was carefully removed to ensure no damage to the slices. Slices were then incubated in increasing concentrations of sucrose (Sigma) solutions (10% and 20% in PBS) for 1h each at RT. Slices were kept in a 30% sucrose solution at 4ºC until further use, with the culture tray covered in parafilm to minimize exposure to the environment.

10.6.2 Immunohistochemical staining

Slices were removed by cutting the surrounding membrane of the porous insert and placed on a microscope slide where the slices were surrounded with DAKO pen (DAKO, Denmark) to protect staining areas from drying. Several PBS washes for the period of 10 minutes occurred before tissue permeabilization with blocking solution (15% BSA, 10% HS in PBS) containing 1% Triton X-100 (Sigma-Aldrich) for a period of 3 hours. The blocking solution was removed and simultaneously the primary antibody (see **Table 10.2**), diluted in blocking solution without 1% Triton X-100, was added to the slices for an overnight period in a humid dark box at 4^{o}C. On the following day, the solution was removed and washed with PBS containing 0.1% Tween-20 (PBST) three times for the duration of 10 minutes each.

Table 10.2 | Primary antibodies used in immunohistochemistry assays. The host animal, as well as the manufacture and the working dilution are indicated. GFAP, Glial Fibrillary Acidic Protein; Iba1, Ionized Calcium Binding Protein – 1

Antibody	Host	Manufacture	Working Dilution
GFAP	Mouse	Milipore	1:500
Iba1	Goat	Abcam	1:1000

The relevant secondary antibody (see **Table 10.3**) was diluted in blocking solution lacking Triton X-100 and added to each slice for 6 hours at RT. At the end, the solution was removed and washed with PBST several times for the duration of 10 minutes each. The nuclei were stained at a dilution of 1:100

with Hoechst 33342 (20µg/ml, Invitrogen) for 40 min at RT. This solution was removed and the slices were washed several times in PBST for the duration of 10 minutes each and mounted in Mowiol.

Table 10.3 | Fluorophore-coupled secondary antibodies used in immunohistochemistry assay. The host animal, as well as the manufacture and the working dilution are indicated.

Antibody	Host	Manufacture	Working Dilution
Alexa Fluor® 488 anti-mouse IgG	Donkey	Life technologies	1:400
Alexa Fluor® 488 anti-goat IgG	Donkey	Life technologies	1:400

10.6.3 Confocal microscopy images

Specific images of CA1, CA3 and DG regions of the hippocampus were acquired with a frame size of 1024 x 1024 pixels on an inverted confocal laser scanning microscope (Zeiss LSM 710) equipped with a Plan-Apochromat 20x objective (Zeiss, Germany) with a numerical aperture of 0.80. Hoechst 33342 fluorescence was detected with a 405 nm diode laser (30 mW nominal output). Alexa Fluor 488 fluorescence was detected using the 488 nm line of an Argon laser (25 mW nominal output). The software used to visualize and obtain the images was ZEN LITE 2012 (ZEISS, Germany). The Z stack option was used with all images to obtain various images from the same location, but at different depths providing images of slices at a set thickness. The images included in this thesis were processed with the orthogonal projection setting set to maximum to provide an image showing the maximum intensities of each of the images of the Z stack.

Images of 40x magnification were obtained in the CA1 region with a Plan-Neofluar 40x objective (Zeiss, Germany) with a numerical aperture of 1.30 with an oil immersion droplet on the lens. A Z-stack was also undertaken and these images were also processed via the same method previously mentioned for the images obtained with the 20x objective.

10.7 FUNCTIONAL ANALYSIS -EXTRACELLULAR FIELD POTENTIALS

The use of electrophysiology studies provides researchers with information on the electrical properties of neurons. This technique measures voltage change or electrical currents and monitors action potential activity by two different types of recordings. The first type is the intracellular recordings, which involve monitoring single ion channel proteins. The second type is the extracellular recordings, that monitor electrical activity of neurons and is the type utilised in this project.

The hippocampus is the most popular region for studying features of neurons due to the well-structured and distinct inputs and outputs. The latter allows a researcher to study the cell membrane potential by injecting current into a cell and monitor how the cell responds. Also, previously mentioned in section 8.2.2, the hippocampal region is useful due to its ability to acquire epileptiform activity. This project monitored the spontaneous epileptiform activity of epileptic-like (EL) organotypic slices; therefore no stimulation with injecting current was used.

Spontaneous epileptiform activity resembles the neuronal activity of seizures and in this study it was recorded with the electrode placed in CA3 region. Discharges can also be viewed in regions CA1 and DG although the CA3 is considered to have more recurring epileptiform discharges in comparison (Rutecki and Yang, 1998). Also, physiologically organotypic slices do not demonstrate any recurring spontaneous epileptiform activity, thus these recordings provide a simple way of comparing slices from both conditions.

10.7.1 Epileptiform field recordings

The epileptiform activity of organotypic slices was recorded in their growth medium using an interface type chamber. This system tends to be the best method, as described in previous studies (Berdichevsky et al., 2012; Dyhrfjeld-Johnsen et al., 2010). This chamber allows the culture medium to pass over the slices, unlike immersion chambers where the slices would be completely immersed in the solution/medium.

At 14 DIV, individual organotypic slices from each of the conditions studied, were transferred to the interface recording chamber with a humidified gas atmosphere (95% O_2 / 5% CO_2) and a temperature of 36^{o}C. The NBA serum free medium (see section 10.2), was continuously superfused and recirculated at a rate of 2mL/min, allowing the slices to have contact with fresh medium.

Following a 20 minute equilibration period, field potential recordings were performed with an extracellular microelectrode (4M NaCl, 2-4MΩ resistance) positioned in the CA3 pyramidal cell layer for approximately 30-40 minutes. Continuous recordings were obtained with Axoclamp 2B amplifier and digitalised (Axon Instruments, Forster City, CA, USA). For data analysis, Clampex software version 10.2 (Molecular Device Corporation, CA, USA) was utilised. All recordings were band-pass filtered (eight-pole Bessel filter at 60 Hz and Gaussian filter at 600 Hz).

10.8 Statistical Analysis of Results

All statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). In all figures data are presented as mean +/- standard error of the mean (SEM). Statistical tests were either unpaired T-tests or one-way analysis of variance (ANOVA) followed by Bonferroni correction post-test. All figures specify the sample size (N) used and the probability (P) values of each data set. Statistical significance was considered when $P<0.05$.

11.1 INFLAMMASOME EXPRESSION IN CONTROL AND EPILEPTIC SLICES AT 14 DIV

Evaluation of NLRP3 expression in CTL and EL slices was obtained through western blot assay, carried out with protein extracts obtained from the hippocampal region of the slices. It is considered that several CNS cells express NLRP3 and these proteins may play both a physiological and pathological role within the cell. Also, an increased expression of this protein may represent the increased activation of intracellular pro-inflammatory pathways and evidence suggests this may be the case with epilepsy (Meng et al., 2014). Activation of NLRP3 is thus considered crucial for initiating the inflammasome assembly.

In relation to the protein bands detected by western blotting with SDS-PAGE, various isoforms have been demonstrated to exist for the NLRP3 inflammasome (Haneklaus et al., 2012). Therefore, the two heaviest bands were selected, since two isoforms are known to exist at these approximate molecular weights.

11.1.1 NLRP3 expression analysis by western blot

The 14 DIV time point was selected to observe differences in protein expression of NLRP3 between CTR and EL slices. At this time point, EL slices already display epileptiform activity therefore making it reasonable to determine any differences.

Both CTR and EL slices express the 106kDa NLRP3 isoform (**Figure 11.1 C**), being this the most expressed in both conditions in comparison to the 118kDa isoform (**Figure 11.1 A)**. The expression of the heavier 118kDa isoform (**Figure 11.1 A)** was only ~12% higher in comparison to the CTR condition, while the 106kDa isoform expression **(Figure 11.1 C & D)** was found to be ~52% higher in comparison to CTR slices. Although EL slices showed a tendency for increased NLRP3 expression, no significant differences ($p<0.05$) were achieved in either 118kDa (CTR: 1.00 ± 0.138 vs EL: 1.120 ± 0.135) or even in the 106kDa (CTR: 1.00 ± 0.174 vs EL: 1.52 ± 0.237) isoforms. However, in EL slices the 106kDa isoform depicts a higher increase in expression, ~40%, in comparison to the increase observed with the 118kDa isoform.

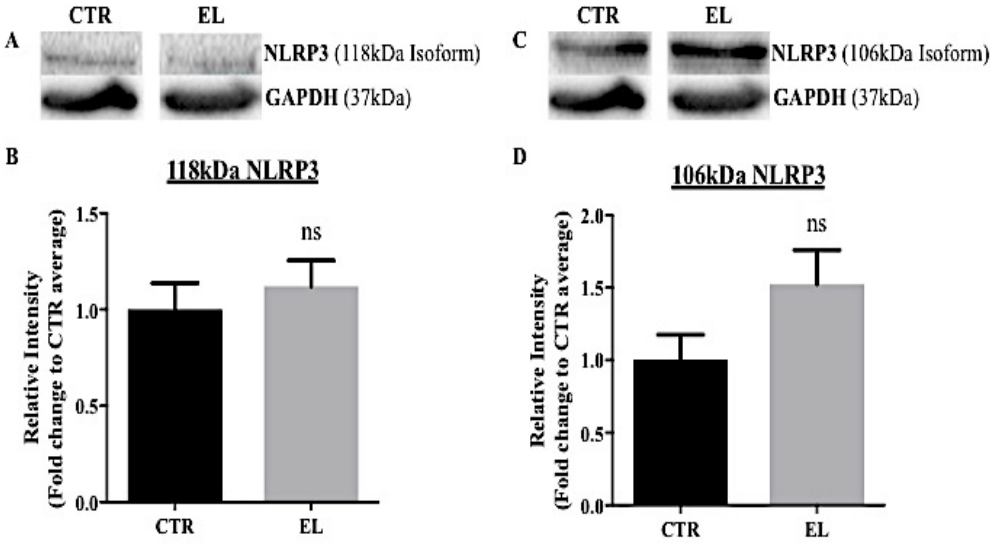

Figure 11.1 | Protein expression of NLRP3 isoforms (118 kDa and 106 kDa) in CTR and EL slices at 14 DIV. (A, C) Representative immunoblots of 118kDa and 106kDa NLRP3 isoforms expression, respectively. **(B, D)** Densitometry analysis of 118kDa and 106kDa NLRP3 isoform expression performed with ImageJ software. Fold change against untreated EL average. All values are mean +/- SEM, N=3 independent cultures. Unpaired T-test showed no significant differences between the groups, $p<0.05$. Statistical tests were performed in comparison with the CTR slices.

11.2 CELL DEATH

As previously mentioned, cell death and neuroinflammation are closely associated. In order to characterize cell death in this slice model of epileptogenesis, an evaluation by αII-Spectrin cleavage was carried out. Spectrin is a protein formed by tetramers of spectrin α and β subunits connected to actin filaments at either end of the tetramer along with other proteins such as cytochrome C. It is located intracellularly in the proximity of the plasma membrane. In the CNS, spectrin is expressed in a variety of cells with neurons being the CNS cell type that expresses this protein most abundantly, such as the αII-spectrin form with 250 kDa (Weiss et al., 2009). In CNS disorders and brain damage this protein is cleaved via calcium sensitive proteases, calpain and caspases, giving rise to spectrin breakdown products (SBDPs). Calpain mediated degradation of αII-spectrin results in the formation of two stable proteins: 150 kDa (SBDP150) and 145kDa (SBDP145). Caspase-3 can form a 150kDa product with it being further cleaved forming 120 kDa (SBDP120; Weiss et al., 2009; Yan et al., 2012). Both 150 and 145 kDa proteins result from necrosis while the 120 kDa product is associated with the process of apoptosis (Weiss et al., 2009).

Therefore, to assay neuronal death, protein expression of SBDPs (except 150 kDa since was poorly visible) against full-length spectrin (FL-Spectrin) expression (250 kDa) was evaluated. These experiments were performed in EL slices at 7 and 14 DIV, treated and untreated with the NLRP3 inhibitor MCC950 for 6 h, to determine if neuronal protection or degeneration was occurring as a result of NLRP3 inhibition.

11.2.1 αII-Spectrin cleavage at 7 DIV

The protein expression of FL-spectrin and SBDPs (**Figure 11.2**) at 7 DIV was observed under all conditions. In the presence of the vehicle (0.1% DMSO) or NLRP3 inhibitor (0.1% DMSO + 10μM MCC950) these showed a tendency for decreased protein cleavage (**Figure 11.2 B & C**).

Relative to SBDP145 formed by calpain cleavage, the graph in **Figure 11.2 B** depicts the ratio between SBDP145 and FL-Spectrin protein expression. Of all three conditions, the vehicle-treated slices demonstrated an average ratio (0.859 ± 0.188) that was the lowest. In comparison to untreated EL slices (1.00 ± 0.145), it depicts an average ratio that is ~14% lower. MCC950-treated slices demonstrate an average ratio (0.8787 ± 0.2675) that is ~12% lower in comparison to untreated slices and ~2% higher in comparison to vehicle slices. Thus, no statistical difference ($p<0.05$) was obtained between DMSO and MCC950 treated slices. Also, no statistical difference was obtained between these two conditions and untreated slices.

The SBDP120 from caspase-3 cleavage, the graph in **Figure 11.2 C** depicts the ratio between SBDP120 and FL-spectrin protein expression. Similarly, to the SBDP145 and FL-Spectrin ratio (**Figure 11.2 B**) the DMSO-treated slices demonstrated an average ratio (0.5193 ± 0.06839) that was the lowest and ~48% lower than untreated EL slices (1.00 ± 0.485). MCC950-treated slices demonstrate an average ratio (0.5957 ± 0.2245) that is ~40% lower than untreated ones. However, it is ~8% higher than in the vehicle group. No statistical significance ($p<0.05$) was found between these groups of slices.

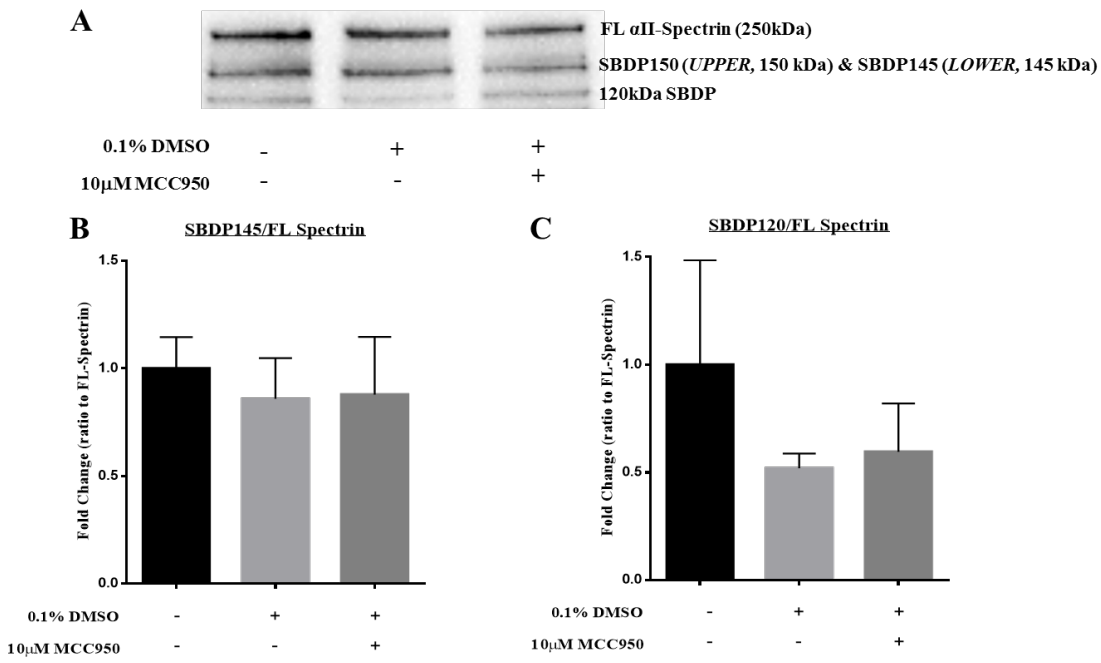

Figure 11.2 | Protein expression of FL-Spectrin and SBDPs of 7 DIV slices under various conditions. A) Representative immunoblot of FL-Spectrin and SBDPSs of 7 DIV slices under various conditions. **B)** Densitometry analysis of 145 kDa protein against FL-Spectrin protein. **C)** Densitometry analysis of 120 kDa protein against FL-Spectrin protein. Fold change against untreated EL average. All densitometry analysis was performed with ImageJ software with the ratio being the SBDP against FL-Spectrin. All values are mean ± SEM, N=3, one-way ANOVA followed by Bonferroni´s Comparison Test.

11.2.2 αII-Spectrin cleavage at 14 DIV

The protein expression of FL-Spectrin and SBDPs (**Figure 11.3**) at 14 DIV was observed under all treatments. In the presence of the vehicle (0.1% DMSO) or NLRP3 inhibitor (0.1% DMSO + 10μM MCC950) these showed a tendency for increased protein cleavage (**Figure 11.3 B & C**).

Relative to SBDP145 calpain cleavage, the graph in **Figure 11.3 B** depicts the ratio between SBDP145 and FL-Spectrin protein expression. Of all three conditions, untreated slices demonstrated an average ratio (1.000 ± 0.3837) that was the lowest. DMSO-treated slices, depict an average ratio (1.309 ± 0.1540) that is ~31% higher than untreated slices. In comparison to slices with vehicle and MCC950 it demonstrates an average ratio (1.159 ± 0.5123) that is ~16% higher in comparison to the standard EL condition. Between the vehicle condition and MCC950-treated slices, the latter had an average ratio that was ~15% lower in comparison. There were no statistically significant differences ($P<0.05$) between any of these conditions.

Relative to caspase-3 cleavage forming SBDP120, the graph in **Figure 11.3 C** depicts the ratio between SBDP120 and FL spectrin protein expression. Similarly to SBDP120 and FL Spectrin ratio (**Figure 11.3 B**) the standard EL condition demonstrated an average ratio (1.000 ± 0.4962) that was the lowest. In comparison to the vehicle condition (1.388 ± 0.1223), it depicts an average ratio that is ~39% higher. While, in comparison to slices in the presence of vehicle and MCC950, it demonstrates an average ratio (1.058 ± 0.5705) that is ~6% lower in comparison to the standard EL condition. Between the vehicle slices and MCC950-treated slices, the latter had an average ratio that was ~33% lower. Meanwhile, there were no statistically significant differences between the conditions.

Meanwhile, there were no significant differences between the vehicle or NLRP3 inflammasome inhibitor conditions.

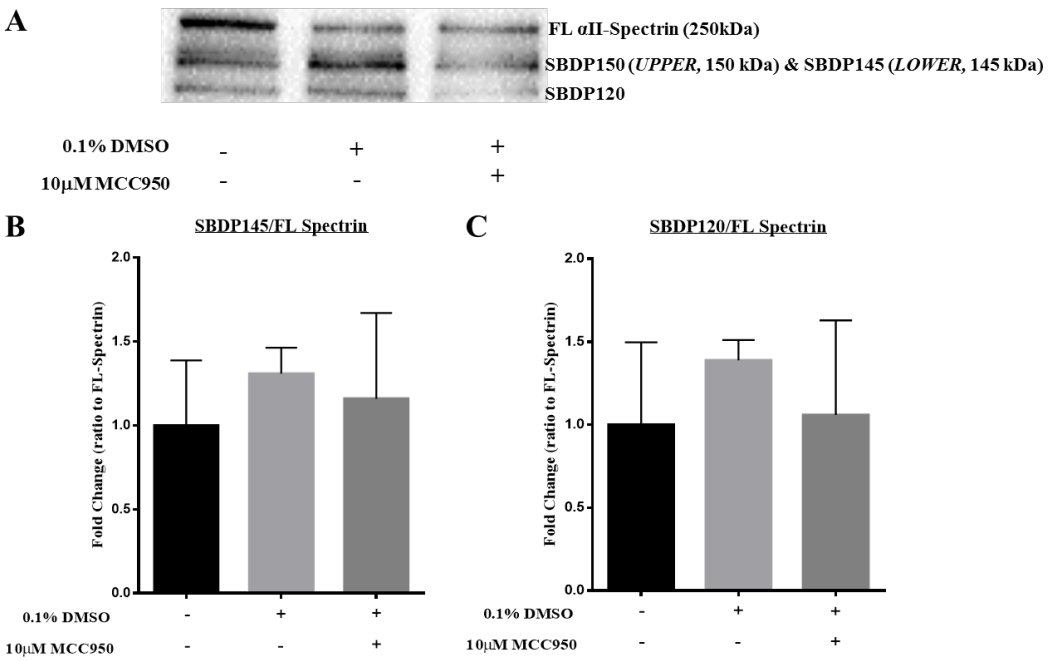

Figure 11.3 | Protein expression of FL-Spectrin and SBDPs of 14 DIV slices under various conditions. A) Representative immunoblot of FL-Spectrin and SBDPSs of 7 DIV slices under various conditions. **B)** Densitometry analysis of 145 kDa protein against FL-Spectrin protein. **C)** Densitometry analysis of 120 kDa protein against FL-Spectrin protein. Fold change against untreated EL average. All densitometry analysis was performed with ImageJ software with the ratio being the SBDP against FL-Spectrin. All values are mean ± SEM, N=3, one-way ANOVA followed by Bonferroni´s Comparison Test.

11.3 ASTROGLIOSIS AND MICROGLIA ACTIVATION

As previously mentioned, one of the main hallmarks of epilepsy is reactive gliosis, a common response to CNS injury, damage and stress. Therefore, the effect of NLRP3 inflammasome inhibition upon this feature was evaluated in the slice model of epileptogenesis.

The glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is specifically expressed in astrocytes. The overexpression of this protein demonstrates the increased proliferation and activation of astrocytes (Sofroniew and Vinters, 2010). The ionized calcium-binding molecule 1 (Iba1) is a cytoplasmic protein that is specifically expressed in macrophages and microglia, and undergoes upregulation during the activation of these cells. This protein is involved in cellular pathways involving calcium and actin reorganizing proteins (Ohsawa et al., 2004). Indeed, it has been demonstrated that actin plays a role in the ability of macrophages and microglia to phagocytose cells and cellular debris (Haberzettl et al., 2007). Thus, Iba1 appears to be vital for microglia and is considered a good microglia marker due to changes in its expression when these cells become activated.

Both GFAP and Iba1 proteins prove to be specific markers for astrocytes and microglia, respectively. In section 11.3.1 and 11.3.2 quantitative analysis of the expression of these proteins was evaluated since it is representative of astrocyte and microglia activation. While in section 11.4, GFAP and Iba1 were used in immunofluorescence assays to assess morphological alterations of astrocytes and microglia under the various treatments.

11.3.1 Protein expression of GFAP at 7 and 14 DIV

GFAP (**Figure 11.4 A & C**) was expressed at 7 DIV and 14 DIV slices in all conditions.

In the presence of DMSO or MCC950 7 DIV slices showed a decreased GFAP expression (**Figure 11.4 A & B**). In DMSO-treated slices, the average GFAP expression significantly decreased (** $p<0.01$) in comparison to untreated ones (EL: 1.00 ± 0.151 vs DMSO: 0.273 ± 0.060). Moreover, MCC950 exposure lead also to a significant decrease (** $p<0.01$) of GFAP expression when compared to untreated slices (EL: 1.00 ± 0.151 vs MCC950: 0.243 ± 0.0733). However, no significant differences were found between DMSO- and MCC950-treated slices.

In 14 DIV slices the presence of DMSO does not affect GFAP expression in relation to untreated slices (EL: 0.970 ± 0.0270 vs DMSO: 1.020±0.135) (**Figure11.4 C & D)**. MCC950 exposure tends to decrease GFAP expression (MCC950: 0.633 ± 0.181), but without relevant statistical differences ($p<0.05$).

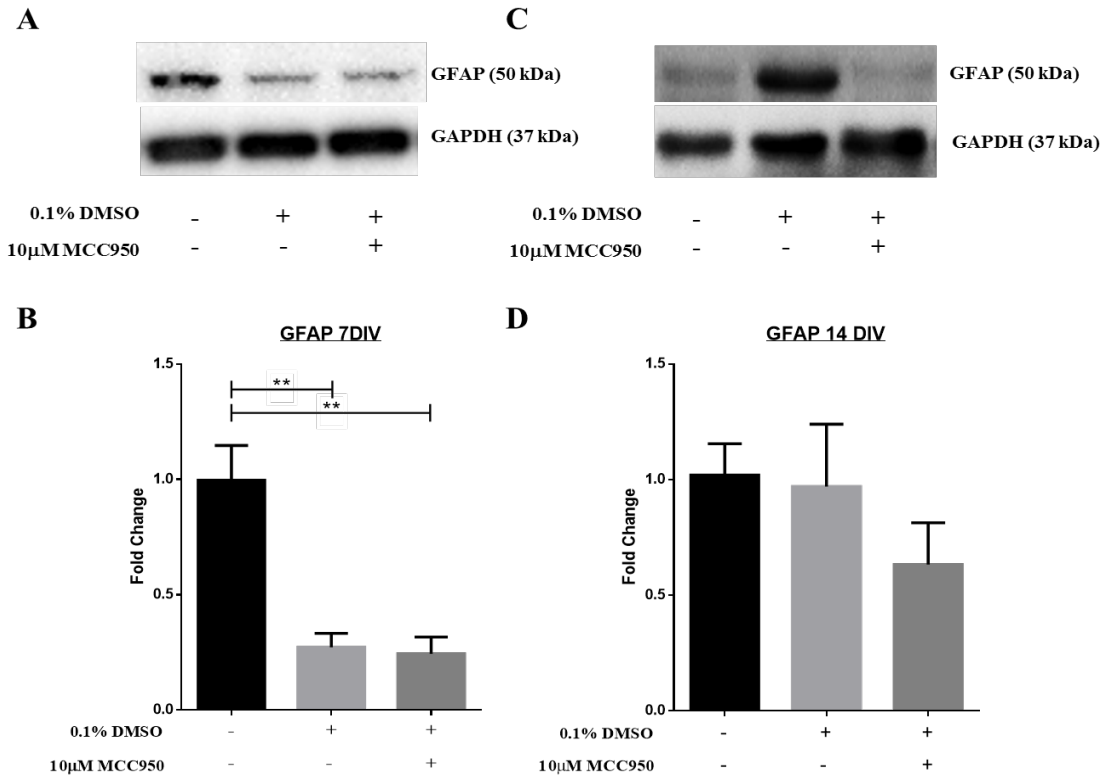

Figure 11.4 | Protein expression of GFAP under various conditions at 7 and 14 DIV. (A, C) Representative immunoblots of GFAP expression at 7 and 14 DIV slices under various conditions. **(B, D)** Densitometry analysis of GFAP expression performed with ImageJ software. Fold change against untreated EL average. All values are mean ± SEM. 7 DIV, N=3-4. 14 DIV, N= 3-5. ** $p<0.01$, one-way ANOVA followed by Bonferroni´s Comparison Test.

11.3.2 Protein expression of Iba1 at 7 and 14 DIV under various conditions

Iba1 expression (**Figure 11.5 A & C**) was observed at 7 DIV and 14 DIV slices in all conditions.

In relation to untreated 7 DIV slices (1.02 ± 0.260), DMSO does not alter Iba1 expression (1.016 ± 0.260). In the presence of the NLRP3 inhibitor, slices show a tendency for decreased Iba1 expression (0.843 ± 0.0482) (**Figure 11.5 B**) in relation to either untreated or DMSO-treated slices, but without statistical significance ($p<0.05$).

In comparison to untreated 14 DIV slices (1.00 ± 0.182), DMSO does not affect Iba1 expression (1.037 ± 0.287) (**Figure 11.5 D**). MCC950 exposure decreases Iba1 expression (0.580 ± 0.0557) (**Figure 11.5 D**), in comparison to either untreated or DMSO-treated 14 DIV slices. But, as happened in 7 DIV slices, these changes were not significantly different ($p<0.05$).

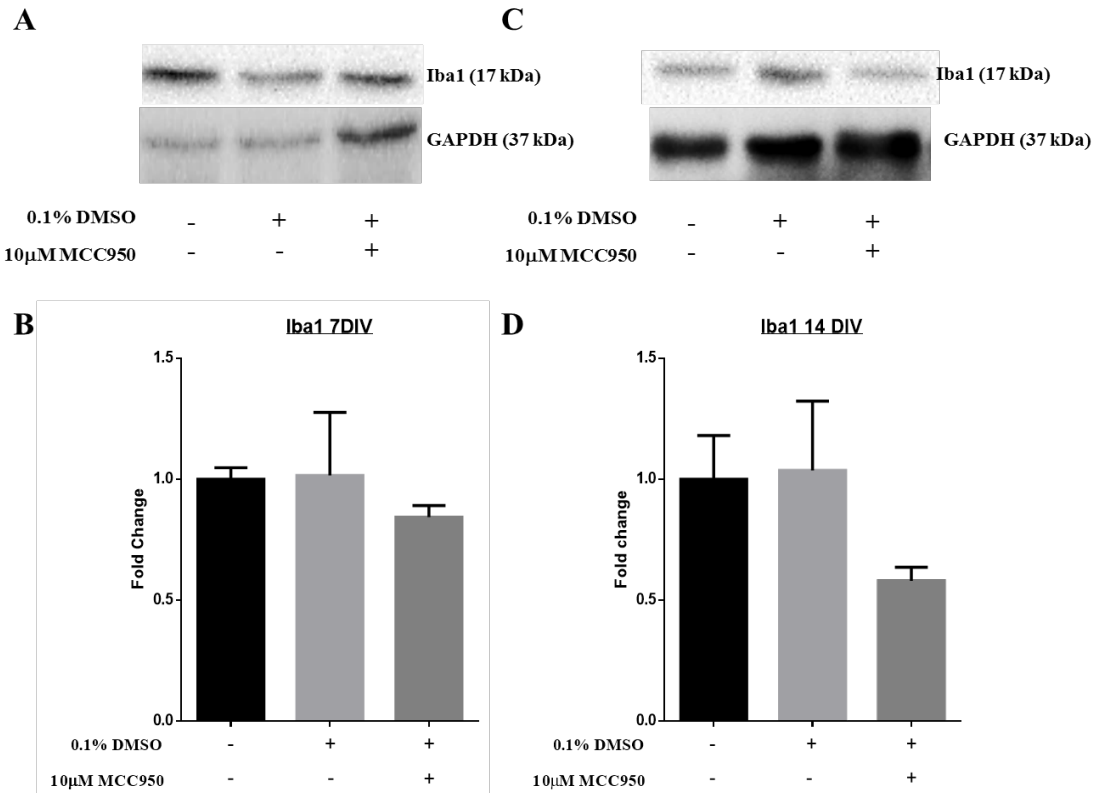

Figure 11.5 | Protein expression of Iba1 under various conditions at 7 and 14 DIV. Representative immunoblots of Iba1 expression at 7 and 14 DIV slices under various conditions. **(B, D)** Densitometry analysis of Iba1 expression performed with ImageJ software. Fold change against untreated EL average. All values are mean ± SEM, 7 DIV, N=3. 14 DIV, N=3. One-way ANOVA followed by Bonferroni´s Comparison Test.

11.4 ANALYSIS BY IMMUNOFLUORESCENCE

The panels of both **Figure11.6** & **11.7** depict the immunofluorescence images obtained by the double detection of Hoechst along with either GFAP (**Figure 11.6**) or Iba1 (**Figure 11.7**). An evaluation of glia morphology under the various conditions at both 7 and 14 DIV was observed in the hippocampus regions DG, CA3 and CA1. Astrocyte activation involves an increase in processes extending from the cell (Sofroniew and Vinters, 2010). While microglial activation, results in the microglial cell reducing its extending processes and increasing the size of its central cell body (Lively and Schlichter, 2013). The morphology of both astrocytes and microglia upon activation are explained in section 8.4.

11.4.1 Astrocyte activation

The GFAP staining in EL slices (**Figure 11.6 A**) showed a vast number of astrocytes with long and overlapping processes that resembles the morphology of the astrocytic glial scar (previously mentioned in section 8.4). This astrocytic scar is clearly defined throughout all regions of the hippocampus. The 40x objective image of astrocyte bodies shown in **Figure 11.6 A4** demonstrates astrocytes with a large number of individual processes arising from the cell body. In the presence of vehicle (**Figure 11.6 B**), hippocampal regions demonstrated a similar large number of astrocytes and morphology as seen in all image panels of EL slices (**Figure 11.6 A**). However, the CA1 region demonstrated a less dense astrocytic scar with cell bodies far less hypertrophic, as observed in more detail in the magnified 40x panel of **Figure 11.6 B4**. Upon exposure to 10μM MCC950, an astrocyte scar is still visualized in all image panels (**Figure 11.6 C**). The CA1 region (**Figure 11.6 C3 & C4**), depict a less dense scar with more processes that appear to be thinner in comparison to the rest of the

conditions. Also, the **Figure 11.6 C4** image demonstrates that these astrocytes have fewer processes emerging from each cell body. Thus, suggesting these are mildly reactive and hypertrophic. To summarise, **Figure 11.6** demonstrates the presence of an astrocyte scar throughout all conditions with differences in terms of their morphology.

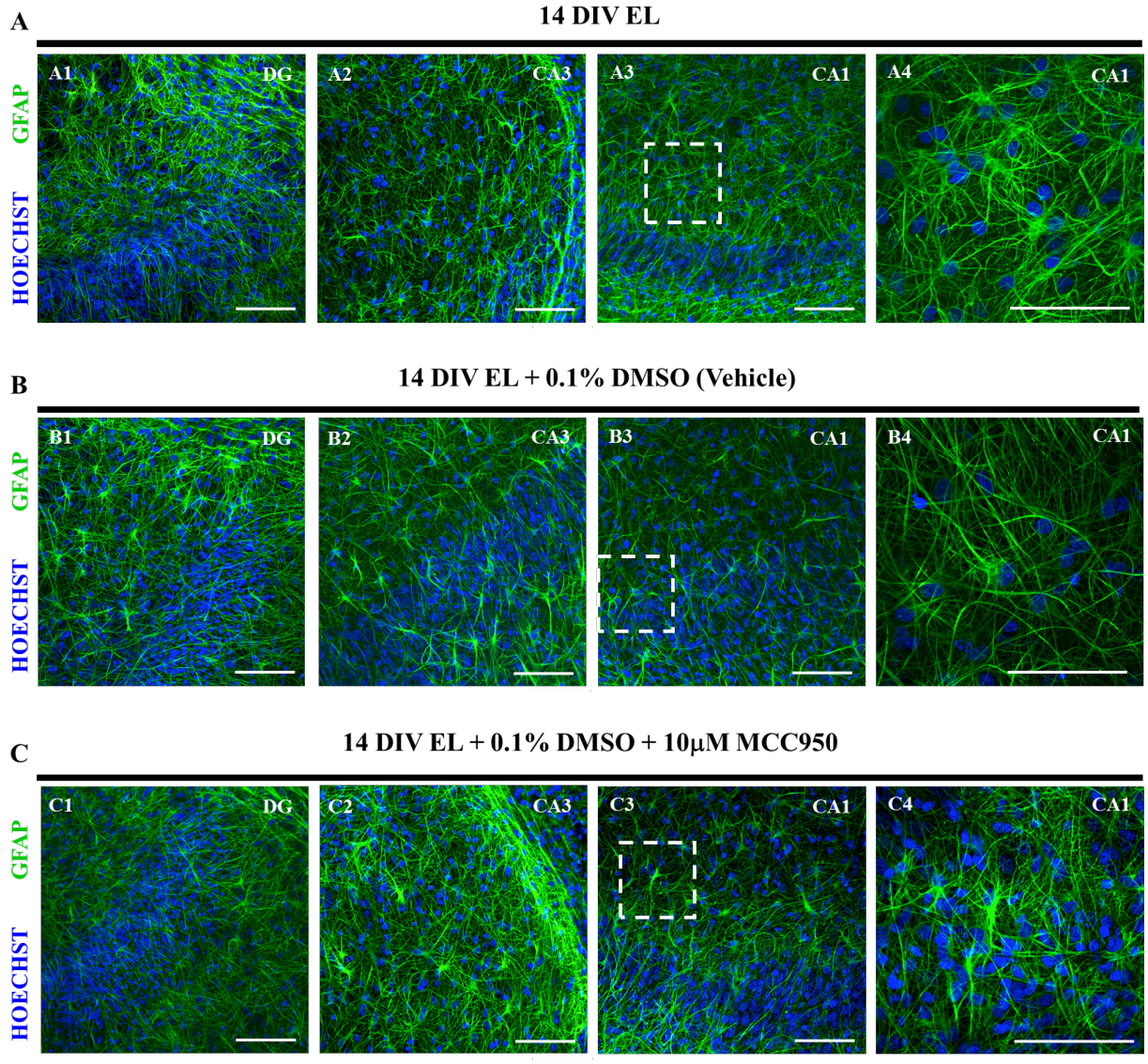

Figure 11.6 | Astrocyte activation in organotypic slices at 14 DIV under various conditions. Detection of Hoechst stained nucleus (blue), together with GFAP stained astrocytes (green). Conditions are indicated on the top of each panel, **A)** EL slices, **B)** DMSO-treated slices, **C)** MCC950-treated slices. The dashed white line represents the area in the CA1 region where the 40x image was obtained (fourth image of each panel). Scale bars (constant white line), 200μm.

11.4.2 Microglia activation

The Iba1 staining in EL slices (**Figure 11.7 A**) showed a large spherical cell shape depicting these microglia being in a reactive form in DG (**Figure 11.7 A1**) and CA3 (**Figure 11.7 A2**) regions. The microglia located in the CA1 **(Figure 11.7 A3)** region display a microglia morphology associating with them being in a prime state due to increased distal ramifications in comparison to DG and CA3 (**Figure 11.7 A1** & **A2** respectively). The 40x objective image of microglia in the CA1 region (**Figure 11.7 A4**) demonstrates these ramifications to be short while they emerge from microglia that appear to have large cellular bodies (indicative of a primed state). In vehicle slices a higher number of microglia were observed in the DG (**Figure 11.7 B1**), CA3 (**Figure 11.7 B2**) and CA1 (**Figure 11.7 B3**) region

compared to that seen in untreated slices (**Figure 11.7 A1-A3**). In terms of morphology, the DG and CA3 region in vehicle slices (**Figure 11.7 B1** & **B2** respectively) demonstrated spherical microglia with cellular bodies being larger in comparison to DG and CA3 in EL slices (**Figure 11.7 A1** & **A2** respectively). Also, the CA1 region (**Figure 11.7 B3**) appeared to depict smaller microglia bodies with thinner ramifications in comparison to those of EL condition. In the presence of 10μM MCC950, slices demonstrated an increased number of microglia in the three regions (**Figure 11.7 C1-C3**) that appears to be similar to the vehicle slices.

In terms of morphology, all regions demonstrated ramified microglia with thinner processes suggesting that MCC950 impairs microglia activation. Indeed, the amplified panel, **Figure 11.7 C4**, depicts microglia with longer and thinner ramified processes resembling the resting state. Also, the central cell body is smaller in comparison to the microglia seen in the vehicle slices.

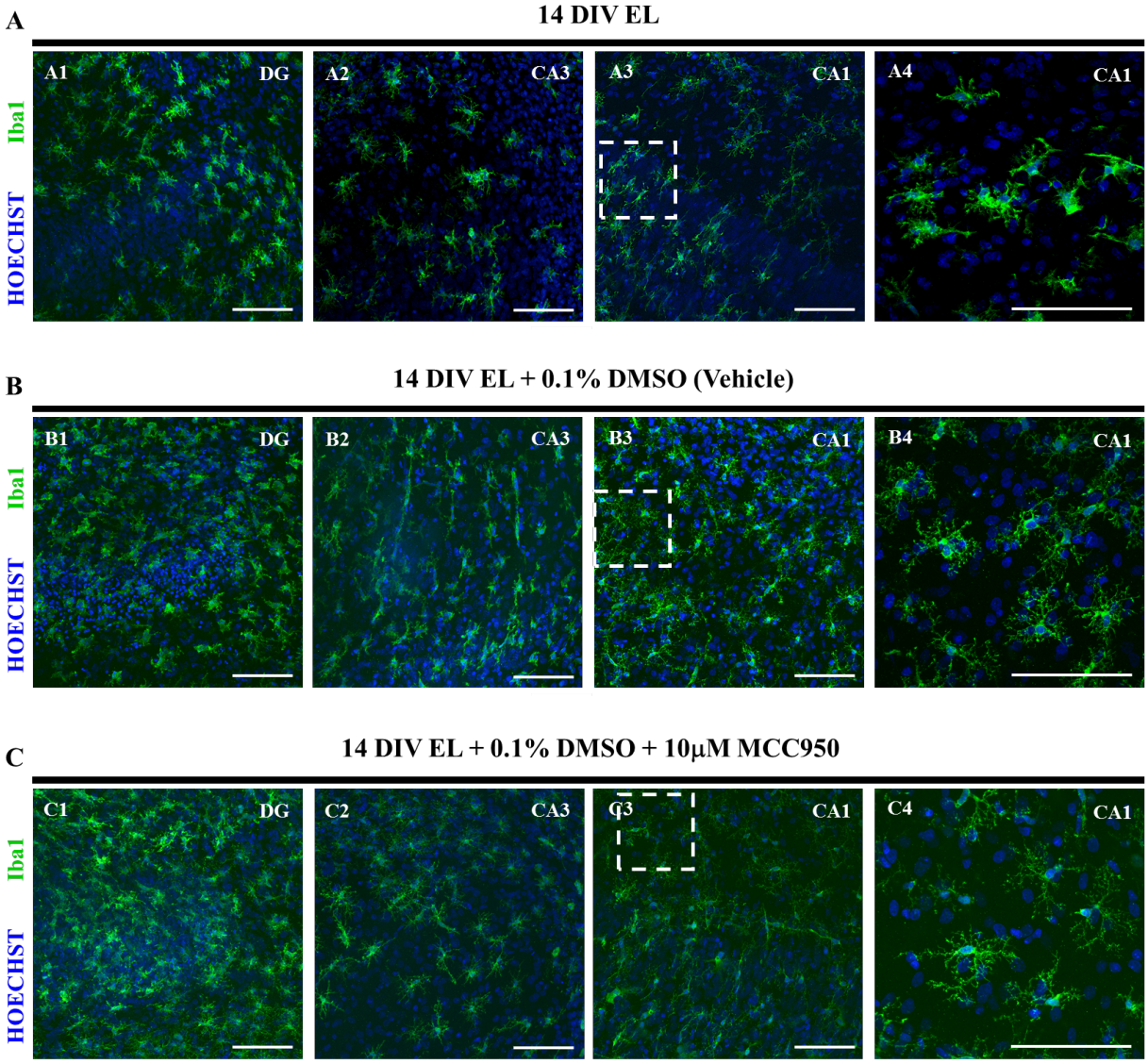

Figure 11.7 | Microglia activation in organotypic slices at 14 DIV under various conditions. Detection of Hoechst stained nucleus (blue), together with Iba1 stained microglia (green). Confocal images were obtained with 20x objective (A1-A3, B1-B3, C1-C3) and a 40x objective (A4, B4, C4). Conditions are indicated on the top of each panel, **A)** EL slices, **B)** DMSO-treated slices, **C)** MCC950-treated slices. The dashed white line represents the area in the CA1 region where the 40x image was obtained (fourth image of each panel). Scale bars (constant white line), 200μm.

11.5 SPONTANEOUS EPILEPTIFORM ACTIVITY OF EL SLICES AT 14 DIV UNDER VARIOUS CONDITIONS

As previously mentioned, 7 DIV slices present little or no spontaneous epileptiform activity, while 14 DIV slices spontaneously develop interictal-like events, which resemble *in vivo* epilepsy. Thus, this model allows to study the effects of NLRP3 inflammasome inhibition, via MCC950 exposure, on the epileptiform activity.

For that, field potential recordings from the CA3 region of 14-17 DIV slices were performed. Vehicle slices were also recorded to determine if there are any implications of 0.1% DMSO on the spontaneous epileptiform activity.

11.5.1 The affect of the various conditions on epileptiform activity

Organotypic slices were exposed to various conditions resulting in vehicle slices and MCC950-treated slices for a 6h period. Following this incubation, slices were transferred to an interface chamber, as explained in section 10.7, and field potentials recordings were undertaken in the CA3 region. The recorded spontaneous activity was characterised based on the type of activity into three classifications. These included (1) no population discharges, (2) interictal-like discharges consisting of single population spikes and (3) mixed interictal and ictal-like discharges characterised as repetitive bursts of population spikes that last <3 minutes (including after discharges). Traces were band pass filtered (eight-pole Bessel filter at 60Hz and Gaussian filter at 600Hz) and the filtered recordings are shown in **Figure 11.8**.

The representative images demonstrated in **Figure 11.8** are preliminary traces of spontaneous epileptiform activity of slices under the various conditions stated in section 10.3. Both EL and vehicle slices **(Figure 11.8 A & B)** depict inter-ictal along with mixed interictal and ictal discharges. The MCC950-treated slices **(Figure 11.8 C)** demonstrated no spontaneous epileptiform activity overall.

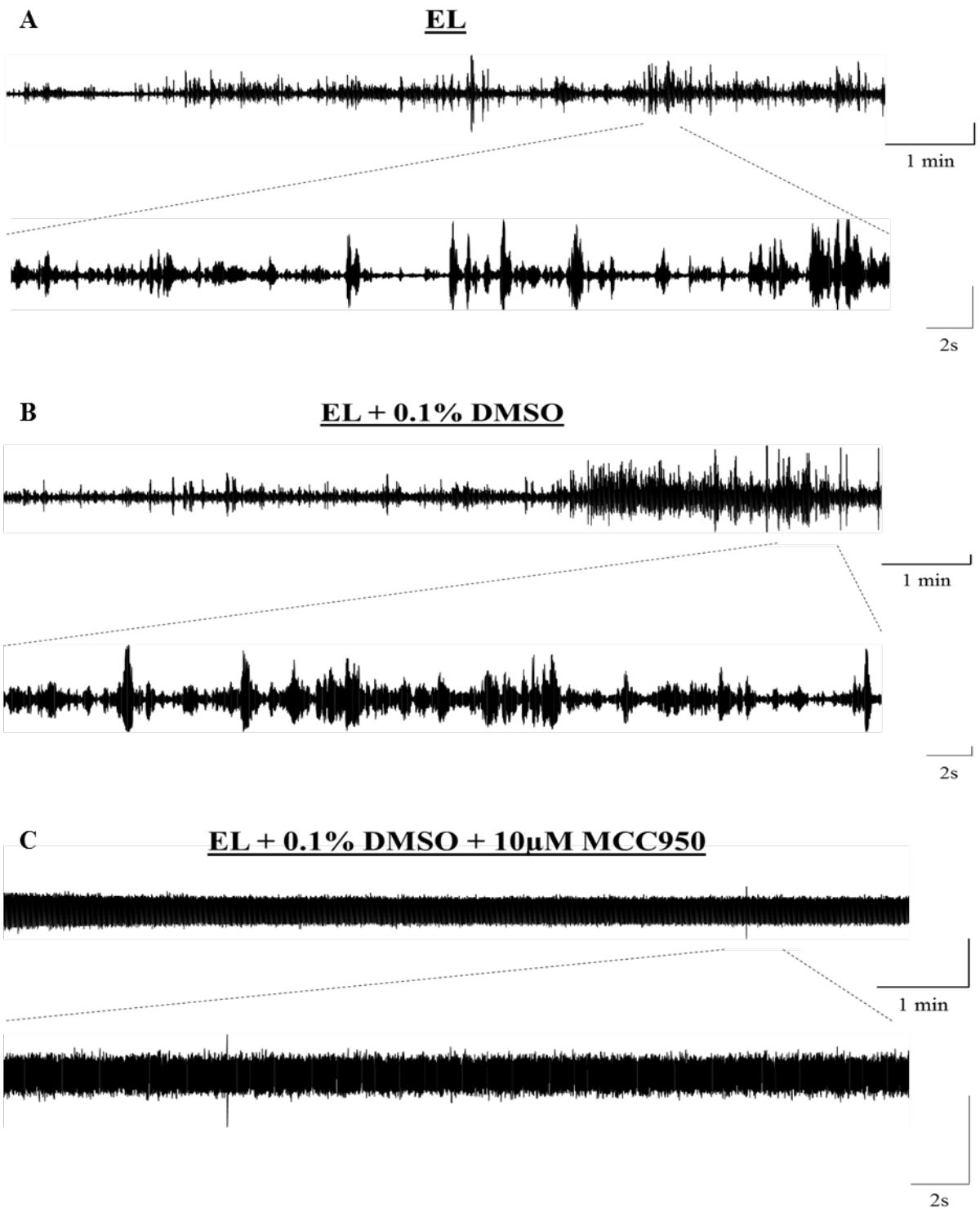

Figure 11.8 | Representative spontaneous epileptiform activity recorded in EL 14 DIV organotypic slices under various conditions. A&B) Interictal, mixed interictal and ictal-like discharges. **C)** No activity**.** All vertical bars = 1mV

12 DISCUSSION

12.1 OHSC AS A MODEL FOR EVALUATING THE ASSOCIATION BETWEEN EPILEPTOGENESIS AND NEUROINFLAMMATION

The study of epileptogenesis is fundamental for understanding the underlying mechanisms for epileptogenesis and discovering new forms of treatment. In this study, the role of the NLRP3 inflammasome was evaluated in a model of epileptogenesis in organotypic slices along with the effect of MCC950, a specific NLRP3 inflammasome inhibitor (Coll et al., 2015). Unlike previous studies, this project focused on the use of a chemical substance, MCC950, instead of reverting to the use of siRNAs (Meng et al., 2014). Also, this study relies on a sterile neuroinflammation to activate glial cells in these cerebral tissues, since it solely has serum free media without the addition of any NLRP3 activating stimuli, such as monosodium urate crystals, etc (Hoffman et al., 2010). This model allows for molecular and functional studies to be undertaken. However, since it is an *ex vivo* model, no evaluation of convulsive behaviour can be assessed (Reddy and Kuruba, 2013). Therefore, this project was aimed at observing the effect of inhibiting NLRP3 inflammasome, on epileptic neural tissue, via evaluating the protein expression and cell morphology of astrocytes and microglia, the immune cells of the brain. Also, the effect of NLRP3 inhibition on αII-Spectrin cleavage and SBDPs production was evaluated since it is considered a marker for neuronal cell death. Finally, electrophysiological recordings allowed for an initial basic evaluation of NLRP3 inhibition on spontaneous epileptiform discharges.

12.2 NLRP3 EXPRESSION IN CONTROL AND EPILEPTIC-LIKE OHSC SLICES

The NLRP3 intracellular receptor located in the cytoplasm of several CNS cells is inactive in the absence of NLRP3 stimuli. The representative figures of NLRP3 expression from western blot analysis demonstrated the 106kDa NLRP3 protein isoform was the most abundant in both the CTR and EL conditions. Previous studies have also evaluated NLRP3 protein expression by analysing the 106kDa NLRP3 protein (Meng et al., 2014). In terms of differences between the CTR and EL slices at 14 DIV, results point to a higher NLRP3 expression in EL slices. However, the results were not significantly different between both conditions in either protein isoform. Therefore, the sample size should be increased to ascertain results that probably represent the global likelihood of these conditions. The trend points to increased NLRP3 protein expression in the 14 DIV EL slices, a time point that depicts spontaneous epileptiform activity. This appears to match the evidence published in studies being either epilepsy or CNS disorders such as Alzheimer's disease, may have increased expression of NLRP3 (Meng et al., 2014; Saresella et al., 2016). Also, the NLRP3 appears to be expressed in the CTR condition and this appears to be a typical occurrence, as a previous study has published evidence of a similar findings along with demonstrating expression of this protein before stimulation occurs (Bauernfeind et al., 2009).

Therefore, in this study if increasing the sample size follows the trend noticed between CTR and EL slices in this study, it may provide further evidence associating both NLRP3 and epilepsy. Also, it may aid in proving the role of neuroinflammation in epileptogenesis, since inflammasomes are a vital component of inflammation while these EL slices depict spontaneous epileptiform activity. Moreover, if a trend is noticed then future studies may want to evaluate if pyroptosis contributes to epileptogenesis (Chen et al., 2016).

This study was limited to analysing NLRP3 protein expression which does not provide sufficient information on other PRRs (especially NLRC4 since it may be expressed in rat astrocytes; Liu and

Chan, 2012) that may be involved, the inflammasome assembly or its activity. Therefore, we are unable to determine the full association between inflammasomes and epilepsy. Future studies could observe protein expression of other PRRs and other types of inflammasome components, including ASC. pro-caspase-1 and caspase-1 (Ahn et al., 2014).

Also, future work should determine if NLRP3 expression alters in the presence of DMSO and MCC950, since this study was unable to do so due to the optimization process required for this primary antibody. It is vital to evaluate this due to MCC950 being NLRP3 specific, thus it specifically prevents NLRP3 inflammasome assembly (Coll et al., 2015). This would depict the MCC950 effect on NLRP3 protein expression (if any) and if other inflammasome components are analysed; this may provide insight on the affect of this compound on NLRP3 inflammasome assembly and/or action.

12.3 NLRP3 INFLAMMASOME INHIBITION AND NEURONAL DEATH

Cell death is one of the hallmarks of epilepsy and evidence suggests either this or cellular degeneration may be crucial for the process of epileptogenesis (Berdichevsky et al., 2013). This study evaluated neuronal death by observing cleavage of the cytoskeletal protein, αII-spectrin (FL-Spectrin, 250kDa), more abundant in neurons than in glial cells. The cleavage occurs by cysteine proteases, such as calpain, mediating the formation of two products, SBDP150 and SBDP 145. While, caspase-3 can form SBDP150 that is cleaved forming SBDP120 (Weiss et al., 2009; Yan et al., 2012). This study assessed the effect of inhibiting NLRP3 inflammasome activation on neuronal death via the ratio between the SBDP and FL-Spectrin. Three groups were thus evaluated: untreated slices, vehicle slices and MCC950-treated slices.

The 7 DIV results demonstrate variations in FL-Spectrin cleavage under the various conditions. These results were not significantly different according to the one-way ANOVA statistical test. Therefore, the sample number should be increased to ascertain the global effect of each condition on neuronal death. Also, this would confirm the trend noticed for 7 DIV slices, which was a decrease in FL-Spectrin cleavage in both vehicle and MCC950-treated slices, in comparison to untreated slices. The trend was not confirmed due to a lack of significant differences between the conditions. However, the protein expression of the SBDP120 product according to WB analysis, demonstrates a considerably large variability in untreated EL slices. Therefore, it may be stated there is no effect occurring between the standard condition and treated conditions. The MCC950-treated slices have a large SEM in comparison to the mean of this group, and it may be speculated that there is a trend in the results between vehicle slices and MCC950- treated slices.

The 14 DIV results demonstrate variations in FL-Spectrin cleavage under the various conditions. These results showed no statistical significance. Therefore, the sample number of all conditions should be altered as stated before for the 7 DIV results. Since, the SEM values of both EL and MCC950-treated slices were considerably large, it may be stated there was no effect since a trend cannot be ascertained. Thus, these results appear to be inconclusive. It would perhaps prove valuable to acquire significant results since this would demonstrate the time point (DIV) when NLRP3 inflammasomes inhibition provides the most neuroprotection (lowering neuronal death).

The vehicle condition used although did not demonstrate any statistically significant differences to the standard EL condition; there are differences between their average ratio values along with some evidence that may result in modifications of future studies using this condition. There are studies demonstrating DMSO promotes neuroprotection, although this data was obtained using higher concentrations of DMSO compared to the 0.1% used throughout this project (Lapuente Chala et al., 2013; Yuan et al., 2014). Evidence suggests this solvent may provide neuroprotection by regulating

the hyperactivity via the neuron-astroglia cytoarchitecture (Lapuente Chala et al., 2013) or reducing neuronal damage (Yuan et al., 2014). Moreover, evidence suggests DMSO can cause widespread apoptosis at a concentration of 0.5% (Hanslick et al., 2009). Thus, there are no published studies demonstrating 0.1% has an affect but due to the results of this study, future studies may want to determine if an affect occurs.

These results with their high variability may be because of experimental error or storage factors. To ascertain the effect, the western blot assay will have to be undertaken again with the same samples to evaluate the effect occurring in these samples. If this proves to provide the same varied results among each condition this may be due to the storage, temperature or thawing having an effect on the degeneration of these proteins (Nardid et al., 1997). Therefore, is this is the case, new samples will have to be analysed with samples that have had limited or no thawing, long period of freezing and exposed to ambient temperature for a shorter period, this may allow results of spectrin cleavage to be observed without such varied results.

Also, another experiment could be undertaken to observe neuronal death as previous studies evaluating the importance of the NLRP3 inflammasome and neuronal death have utilised terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling (TUNEL) microscopy techniques (Meng et al., 2014). This approach detects DNA fragmentation resulting from apoptosis and may also label cells that have suffered severe DNA damage, allowing the researcher to identify neuronal loss via neurons with condensed nuclei. In this case, the researcher will be able to observe neuronal loss in different regions of the hippocampus (or tissues including that from a kidney) and compare them to the surviving neurons (Kádár et al., 2009; Meng et al., 2014). The latter can be observed using Nissl staining, since degrading neurons are associated with loss of Nissl staining (Kádár et al., 2009).

12.4 NLRP3 INFLAMMASOME INHIBITION AND ITS ASSOCIATION WITH ASTROGLIOSIS AND MICROGLIAL ACTIVATION

Studies have determined microglia may express NLRP3 and various inflammasome components (ASC, pro-caspase-1, etc.), while astrocytes are described to contain several inflammasome components and lack NLRP3 expression (Gustin et al., 2015). Therefore, we evaluated the activation of astrocytes and microglia at 2 different DIV to ascertain the affect of NLRP3 inflammasome inhibition at both early and late stages of this epileptogenesis model. Previous studies within this model demonstrated that EL slices at 7 DIV had a reactive microglia phenotype, while these appeared to be in a more resting state at 14 DIV. These studies also demonstrated an increase in intense astrocyte activation (astrogliosis) along with the detection of spontaneous epileptiform activity (Magalhães et al., 2017; in preparation). Both astrogliosis and intense microglia activation (microgliosis) were evaluated in this study via protein expression and morphology studies. Both methods are widely used since they allow evaluation of molecular activation of astrocytes and microglia along with allowing an observation of the shape of the cells in the various regions of the hippocampus. The results demonstrated that both DMSO and MCC950 affected the activation of both astrocytes and microglia.

12.4.1 Astrocyte activation

The 7 DIV slices demonstrated significant differences between the untreated EL slices, vehicle group along with MCC950-treated slices. This demonstrates both DMSO and MCC950 influence astrocyte activation. This time point also demonstrated no statistical difference between the vehicle slices and MCC950-treated slices with the difference between the averages of these conditions being almost non-existent. Therefore, it may be considered that DMSO at this time point is influencing astrocyte

activation. The 14 DIV slices demonstrated no statistical significant differences among conditions in terms of the trends observed, which point to a slight increase in GFAP protein expression in vehicle treated slices, and a decrease in MCC950-treated ones. Thus, a larger sample size is required to determine if the trend observed is valid and would determine if inhibiting NLRP3 inflammasomes have an effect in astrocytic cells.

The confocal microscopy images obtained demonstrated an astroglial scar, which remained throughout all the conditions. The vehicle condition demonstrated a less dense astrocyte scar while in the presence of MCC950 there appeared to depict more astrocytic processes although these appear to be thinner in comparison to the standard EL condition and vehicle condition. Therefore, astrocytes appeared to be less reactive when in the presence of DMSO and/or MCC950.

The results with DMSO previously mentioned may be due to several factors since studies have focused on its effects on astrocytes (Lapuente Chala et al., 2013; Yuan et al., 2014; Zhang et al., 2017). Evidence depicts mixed effects of DMSO on astrocytes being induces reactive gliosis (Zhang et al., 2017), neuronal protection via interacting with glial cells and neurons (Lapuente Chala et al., 2013) and causes upregulated apoptosis of astrocytes (Yuan et al., 2014). In comparison, this model demonstrates DMSO has the ability to decrease neuroinflammation by reducing the active morphology and expression of GFAP. The trend in these results has been previously noted by researchers (Lapuente Chala et al., 2013). However, this research group also noted that low concentrations similar to that used in this project had no effect on astrocytes. Therefore, it may be assumed if the trend in the results collected from this model appears to demonstrate the model is susceptible to DMSO effects at low concentrations. Therefore, future studies should probably use a different solvent (examples mentioned in section 10.4) as the vehicle allowing for a more insightful evaluation of the effect of MCC950.

Also, it is considered astrocytes do not express NLRP3, with it being assumed any present NLRP3 in astrocytes is from microglia that are present in astrocyte cultures due to inefficient astrocyte isolation (Gustin et al., 2015). Therefore, it should be determined how NLRP3 expression decreases GFAP expression decreases

12.4.2 Microglia activation

The 7 AND 14 DIV slices under the three conditions demonstrated no statistical significance between them. Thus, the sample size will have to be increased to determine the significance of the trend noticed in the effect caused by DMSO and/or MCC950. The vehicle condition for the epileptic slices at both DIVs demonstrated a slight increase that appeared to vary more in EL 14 DIV slices due to the high SEM value. Therefore, it may be considered this demonstrated little or no differences between these conditions. The MCC950-treated condition demonstrated Iba1 protein expression decreased to a level that was less than the other conditions at both time points. The 14 DIV EL slices in the presence of the NLRP3 inflammasome inhibitor compared to the 7 DIV time point demonstrated the highest difference verses the standard EL and vehicle conditions.

The confocal microscopy images obtained demonstrated both the vehicle slices and MCC950-treated slices depicted effects on the morphology and number of microglia cells. Both of these conditions appeared to increase the number of microglial cells compared to the standard epileptic like condition. The images of the DG region demonstrated the condition containing MCC950 appeared to have the largest number of microglial cells with all three conditions demonstrating an active morphology (large cell bodies with little or no processes). The CA3 and CA1 region appear to have less microglia in the condition with the presence of MCCC950 compared to the other conditions. In terms of morphology, MCC950-treated slices demonstrate microglia that have the longest processes and smallest cell bodies compared to the other conditions (vehicle condition appears to be less active in comparison to EL)

therefore it may be considered these are in the resting state. While the DMSO condition depicts microglia that may be in a primed state.

Thus, this study suggests inhibiting the NLRP3 inflammasome has consequences on microglia activation. However, no statistical significance was achieved with Iba1, a diminishing trend of microglia specific marker expression and resting state morphology was observed in MCC950-treated slices.

However, the sample size should be increased, allowing for comparison in the number of microglia cells, characterization of processes and cell body size as previous studies have conducted (Cerbai et al., 2012). Another important feature would be the study of cell to cell size ratio due to strong correlation between these and activated microglia (Hovens et al., 2014). It is highly recommended future studies conduct analysis via including these methods especially when using compounds that may effect their morphology. It was unable to be conducted in this experiment due to not having a sample size larger than one. Thing and using the same techniques for IHSC and confocal microscopy, it would be possible; although, the method would have to use the same depth and location to ensure unbiased results.

It has been determined NLRP3 along with several other inflammasome components (proteins) are expressed in rat microglia (Gustin et al., 2015). Future studies should determine NLRP3 localisation and quantity in future work as mentioned in 12.4.1. This would allow an assessment of the microglia changes and NLRP3 to determine any type of association or correlation with limited microglia activation. Also, the vehicle condition demonstrated 0.1% DMSO has little or no effect on Iba1 protein expression but may have an effect on the morphology of 14 DIV EL slices. Therefore, future studies may want to change the solvent used as a vehicle.

12.5 IS NLRP3 ACTIVATION ASSOCIATED WITH THE ONSET OF EPILEPTIFORM DISCHARGES IN THIS OHSC EPILEPTIFORM MODEL

An advantage of this epileptogenic model is the ability to use electrophysiological field recordings to observe spontaneous epileptiform activity. Previous studies have used OHSC models and been able to observe electrophysiological activity. This study used field recordings from the CA3 region of the hippocampus to ascertain the spontaneous epileptiform activity (if any) of slices from the three conditions focused on in this study.

The electrophysiological traces obtained demonstrated inter-ictal along with mixed interictal and ictal discharges in the untreated EL and vehicle slices. These are associated with typical electrophysiological traces obtained during seizures (ictal) and between seizures (inter-ictal; Berdichevsky et al., 2012). The MCC950-treated slices did not depict any spontaneous epileptiform activity in the trace obtained (this would have to be determined by more electrophysiological experiments).

Since only preliminary results of electrophysiological traces of these conditions were obtained, it is required for these experiments with a higher sample size to be undertaken to determine the specific effects of MCC950 upon spontaneous epileptiform activity and ensure there is no spontaneous epileptiform activity. Also, increasing the sample size shall determine the effects of DMSO (if any) on these OHSC slices since comparisons cannot be currently undertaken.

Since there is the possibility that each organotypic slice will vary, to study the effects of the compound on each slice instead of each culture, modifications should be made. Therefore, spontaneous epileptiform activity of an EL slice should initially be analysed by electrophysiological recordings

until a constant stable trace is detected (>10 minutes). While still undertaking the recordings, the drug should be added to the recirculating media at the respective concentration and the time noted. Thus, any differences from this time point can be evaluated by comparing the conditions of a decent sample size. This technique would also demonstrate the time that may be taken for an effect to occur and remove some biasness from the results.

12.6 THE IMPORTANCE OF EVALUATING CYTOKINE EXPRESSION AND RELEASE ESPECIALLY FOR FUTURE RELATED STUDIES INVOLVING INFLAMMASOMES

Cytokines are considered to be associated with epileptogenesis since they appear to be modulated by one and another. The most well studied cytokine is IL-1β (see section 8.5.2.1) due to it being one of the pro-inflammatory mediators in neurodegenerative diseases. As previously mentioned, this cytokine is processed by caspase-1 with this event being triggered by the various inflammasomes including the NLRP3 inflammasome (Martinon et al., 2002).

Therefore, cytokine expression and release should have been evaluated to determine if NLRP3 inflammasome inhibition in the epileptogenic model decreased cytokine levels (such as IL-1β and IL-18). Previous studies have evaluated cytokine levels in regards to inflammasome activation since researchers believe this to be an indicator of inflammasome assembly and function (Coll et al., 2015). Thus, can use approaches such as enzyme-linked immunosorbent assay (ELISA) techniques that relies on using antibodies complementary to the desired substrate followed by a colour change that can be quantified (Lequin, 2005). Determining IL-1β levels are necessary since an upregulation of this cytokine may demonstrate successful inflammasome assembly and function (Coll et al., 2015). Thus, this would provide insight into successful or unsuccessful inflammasome assembly especially if other inflammasome components (ASC, etc.) are evaluated (Ahn et al., 2014). Also, in terms of using rat brain tissue, this may provide some indication if NLRP3 inhibition is sufficient to decrease IL-1β levels especially when the rat astrocytes express NLRC4 inflammasome that have the ability to also activate caspase-1 and process IL-1β (Chen et al., 2014).

Finally, quantifying cytokines such as IL-1β may provide more information on pyroptosis since this cytokine is associated with the occurrence of this form of cell death (Miao et al., 2010).

12.7 IS MCC950 A VIABLE SUPPRESSOR OF NEUROINFLAMMATION AND RECURRING EPILEPTIC DISCHARGES?

This study provided some insights into the effects caused by MCC950 of which the results show to be promising. However, these results should be further experimented to gain statistical significance, since this was a preliminary study. In terms of neuroinflammation, the changes observed in astrocyte and microglia morphology appear to show that MCC950 has an effect at preventing gliosis, that is further corroborated by the decrease in GFAP and Iba1 expression.

Therefore, this study has demonstrated MCC950 to be promising as a viable suppressor of both neuroinflammation and recurring epileptic discharges. To determine its viability and efficacy upon diminishing epileptic recurring discharges and neuroinflammation, MCC950 needs to be evaluated in more epileptogenic models that depict aspects of epilepsy, not depicted in this *ex vivo* model, including convulsive behaviour. Also, the more epileptogenic models used, more insights into the efficacy of MCC950 will be achieved.

13 CONCLUSION AND FUTURE PERSPECTIVES

To conclude, this preliminary study demonstrated promising results of MCC950 being a viable suppressor of epilepsy. Also, it provided information on the biology of the disorder since some insights were gained in terms of the association between glial activation (astrocytes and microglia), neuronal death (αII-Spectrin) and epilepsy (epileptiform activity). The results obtained in the *ex vivo* model of epileptogenesis in organotypic slices, demonstrated that NLRP3 expression was higher in EL slices compared to CTR slices. Expression of αII-Spectrin and its cleavage products was evaluated after incubation with MCC950 (10μM) over a period of 6 hours, with no effect observed. On the other hand, exposure to MCC950 (10μM) caused a decrease in Iba1 and GFAP expression, and changes in astrocytes and microglia morphology obtained by confocal microscopy. In addition, the slices exposed to MCC950 did not demonstrate epileptiform activity obtained by spontaneous field potency records from the hippocampal CA3 region. Thus, MCC950 seems to silence the recurrent epileptic discharges present in EL slices.

Future studies should increase the sample number for the experiments undertaken in this project to determine the precise effect of MCC950. Another assay that should be performed is cytokines' quantification by ELISA, to assess if NLRP3 inhibition as any effect upon the expression and release of the main pro-inflammatory cytokines, specifically IL-1β quantification is particularly relevant since their expression is regulated by NLRP3 (Martinon et al., 2002). Another aspect that could be evaluated to understand the association between inflammasomes and epilepsy would be to evaluate NLRC4, since this has been shown to be expressed in rat astrocytes (Liu and Chan, 2012). Also, this study suggests a contribution of NLRP3-induced pyroptosis for epileptogenesis since there may be differences in NLRP3 protein expression between CTR and EL slices. Thus, an evaluation of the various types of cell death in this *ex vivo* model, namely necrosis, apoptosis and pyroptosis, would also be important.

To evaluate the efficacy of MCC950 on halting neuroinflammation and epileptiform activity it would be recommendable to use other types of epilepsy models including *in vivo* models, since this would provide further evidence for MCC950 halting seizures and epileptogenesis along with providing information on how it effects convulsive behaviour.

Hopefully, future therapeutic approaches will include this compound (if MCC950 is efficient) providing the scientific community with further understanding of the association between neuroinflammation, glia and epileptogenesis.

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